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SIGNAL TRANSDUCTION IN CELL VOLUME REGULATION

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SIGNAL TRANSDUCTION IN CELL VOLUME REGULATION

een wetenschappelijke proeve op het gebied
van de Medische Wetenschappen

PROEFSCHRIFT

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Aan mijn ouders
en oom Hans en tante Ina

Abbreviations

BCECF-AM	2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester
8Br-cGMP	8-Bromoguanosine 3',5'-cyclic monophosphate
Ca^{2+}	calcium
$[Ca^{2+}]_i$	intracellular calcium concentration
cAMP	adenosine 3',5'-cyclic monophosphate
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetra acetic acid
EIPA	ethylisopropylamiloride
FCS	fetal calf serum
fura-2 am	fura-2 acetoxymethyl ester
IBMX	3-isobutyl-1-methylxanthine
HEPES	N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid
KHB	Krebs-Henseleit buffer
pH_i	intracellular pH
PK	protein kinase
PT	proximal tubule
RVD	regulatory volume decrease
RVI	regulatory volume increase
Tris	tris(hydroxy-methyl)aminomethane
TPA	12-O-tetradecanoylphorbol-12-acetate

CONTENTS

CHAPTER 1

General Introduction

GENERAL INTRODUCTION

The ability to keep cell volume constant (cell volume homeostasis) is an important property of most cells as the cell membrane is highly permeable to water [Macknight, 1988; Hoffmann and Simonsen, 1989] As water diffuses easily through the lipid bilayer it will passively follow solute flux [Van Os *et al*, 1994] Therefore, the osmolarity of the cytoplasm and of the extracellular fluids determines cell volume. Alterations in cell volume and as consequence in the concentration of intracellular solutes can interfere with a variety of cell functions which could be lethal for the cell The body fluids of most higher animals have an osmolarity which is isotonic with respect to the cell contents and is maintained within narrow limits (± 3%) by whole bodywater homeostasis [Hoffmann and Simonsen, 1989] Only a limited amount of body tissues, like intestinal epithelial, kidney medullary and blood cells are normally exposed to anisosmotic conditions [Hoffmann and Simonsen, 1989] Nevertheless cell volume homeostasis is also important in tissues which are normally not exposed to variation in extracellular osmolarity

On one hand, cells tend to swell as a consequence of the Gibbs-Donnan equilibrium [Macknight, 1988, Schultz, 1989] as they contain a high concentration of charged macromolecules. On the other hand, changes in intracellular osmolarity will influence cell volume In earlier studies the Na^+/K^+ pump has been defined as the most important system that counteracts swelling by pumping Na^+ out of the cell The combination of passive leak and active pumping is known as the pump-leak hypothesis [Leaf, 1959, Macknight, 1988] From later studies, however, it appeared that a number of other transport systems play an additional role in cell volume regulation and that this process is more complex than initially anticipated [Hoffmann and Simonsen, 1989, Sarkadi and Parker, 1991] The passive flux of solutes and water across the plasma membrane was found to be more dynamic and the intracellular osmolarity is liable to changes in several ways.

Volume regulatory systems are also important in polarized epithelial tissues that mediate large transcellular solute and water fluxes The in- and efflux mechanisms present in the apical and basolateral membrane have to be in exact balance in order to prevent changes in cell volume Sudden fluctuations in metabolic rate of a cell may also result in changes in the concentration of intracellular solutes and, therefore, cell osmolarity [Lang *et al*, 1993] In

addition, activation of ion transport by hormones, growth factors, changes in cell membrane potential or intracellular pH (pH_i) can influence the intracellular osmotic composition of the cell and in this way might activate volume regulating mechanisms [Hoffmann and Simonsen, 1989; Lang *et al.*, 1993]. Besides, Haussinger and Lang [1992] have shown that cell volume is involved in the regulation of hepatic metabolism. Insulin produces cell swelling in both isolated perfused rat liver and isolated rat hepatocytes while glucagon resulted in cell shrinkage Cell volume was found to be a potent modulator of hepatocyte function since hyposmotic liver cell swelling resulted in an anabolic reaction by inhibition of glycogenolysis and proteolysis and a simultaneously stimulation of glycogen and protein synthesis The opposite was observed after hypertonic cell shrinkage which resulted in a catabolic reaction. These results suggest that insulin and glucagon carry out their function in hepatocytes by altering cell volume. Furthermore, conditions that decrease the energy state of the cell, like ischemia, can directly or indirectly influence the activity of transport mechanisms and in this way also result in cell volume changes [Anderson *et al.*, 1990]. The presence of volume regulatory mechanisms could mean the difference between cell injury or recovery after ischemia Finally, cell volume regulation will be of importance in cell growth and proliferation where a balanced increase is needed in intracellular solutes, cell volume and cell surface area [Hoffmann and Simonsen, 1989] In conclusion, in most tissues of the body, cell volume regulation is needed to compensate for volume perturbations caused by variation in intracellular osmolarity

Normally, transport systems involved in cell volume maintenance are operating at a low rate and cell volume perturbation using anisosmotic solutions is often used to activate these systems [Hoffmann and Simonsen, 1989]. Many studies have indicated that a wide variety of cells are able to regulate their volume in anisosmotic solutions [Eveloff and Warnock, 1987; Macknight, 1988; Hoffmann and Simonsen, 1989]. In many invertebrates, cell volume regulation is accomplished by altering the content of organic solutes, for example amino acids, carbohydrates or urea after osmotic cell volume perturbation [Chamberlin and Strange, 1989] In contrast, in mammals transport proteins like exchangers, cotransporters and ion channels which influence the cellular content of inorganic ions are mainly used to regulate cell volume after anisosmotic incubation [Chamberlin and Strange, 1989, Sarkadi and Parker, 1991]. The subject of this thesis is restricted to volume regulation of mammalian cells, isolated from rat and rabbit, involving primarily transport of potassium, chloride and sodium in and out

of the cell

After incubation of cells in hypotonic media swelling does occur and almost all cells that have been studied try to restore their volume towards basal values, a process which is called regulatory volume decrease or RVD [Eveloff and Warnock, 1987; Chamberlin and Strange, 1989, Hoffmann and Simonsen, 1989] The mechanisms involved in RVD depend on the cell type under study and the species it originates from [Macknight, 1988; Lang *et al.*, 1993] Also when cells are shrunk after incubation in hypertonic medium several cell types studied restore their cell volume via a process named regulatory volume increase or RVI As with RVD, the mechanisms involved in RVI appear to be cell specific [Lang *et al.*, 1993]. Some cells only show a so-called pseudo-RVI or post RVD-RVI meaning that they increase their volume after cell shrinkage only when they are pre-incubated in a hypotonic medium and underwent RVD [Hoffmann and Simonsen, 1989; Lewis and Donaldson, 1990] The most important transport systems involved in RVD and RVI will be discussed later in more detail In this thesis focus will be on RVI after hypertonic cell shrinkage as this process has been studied less extensive as RVD

Cell volume regulation

The process of cell volume regulation is not understood in great detail despite its importance for proper cell functioning Diluting or concentrating components involved in cellular signal transduction processes might severely disturb cell functioning. Most studies investigating cell volume regulation have based their conclusions on the behaviour of mean cell volume and mean ion transport rates, determined in cell suspensions [Kimelberg *et al.*, 1992] The regulation of the activity of a certain transporter and its consequence for cell volume will be better understood when both parameters are studied simultaneously at the single cell level. Only in recent years it has become feasible to measure changes in the concentration of intracellular ions like Ca^{2+}, H^+, Na^+ and K^+ in single cells in a more accurate way using fluorescent ratio probes [Haugland, 1992] Studies in which intracellular calcium concentration ($[Ca^{2+}]_i$) is measured in single cells have indicated that there is a heterogeneity in Ca^{2+} homeostasis among individual cells [Willems *et al* , 1993, Wiltink *et al* , 1993] and similar heterogeneity may also exist in cell volume regulatory processes. It would, therefore, be interesting to combine measurements of single cell volume with measurements of second messengers like $[Ca^{2+}]_i$ and pH_i and the activity and regulation of specific

transporters. Therefore, in this thesis volume changes were studied which can be derived from alterations in the concentration of a fluorescent dye in single renal epithelial cells. This method was compared with two other methods for measuring cell volume.

The process underlying cell volume regulation can roughly be divided into three phases. Initially, a cell has to sense the change in volume, subsequently this signal has to be processed and finally cell recovery systems have to be activated. These steps in cell volume regulation will be explained in more detail in the following paragraphs.

First, changes in volume will have to be detected either by a separate volume sensor or in the case of an ion channel this could be an intrinsic property of the transporter itself. Changes in several components that could serve as a volume sensor have been proposed [Sarkadi and Parker, 1991]. Up to now for none of them there is hard experimental evidence, but several studies indicate that the cytoskeleton is a promising candidate [Mills, 1991; Linshaw *et al.*, 1992]. Membrane tension after cell swelling might be mediated through the cytoskeletal network and subsequently open stretch activated ion channels [Sackin, 1987] or alternatively close stretch inactivated ion channels [Morris and Sigurdson, 1989] For detection of cell shrinkage a role of the cytoskeleton seems less obvious and experiments carried out by Foskett and Spring [1985] demonstrate no change in RVI in gallbladder epithelium after treatment with agents that interfere with the integrity of the cytoskeleton. A mechanism which enables the cell to detect both cell swelling and cell shrinkage and which would directly interfere with signal transduction is a phenomenon called macro molecular crowding. A high concentration of non-reactive macromolecules can alter the specific activity of certain enzymes [Sarkadi and Parker, 1991; Parker, 1993; Garner and Burg, 1994]. Since kinases or phosphatases modulate several transport mechanisms which are involved in cell volume regulation [Grinstein *et al.*, 1992; Klein *et al.*, 1993], it is feasible that the activity of these kinases and phosphatases is directly regulated by swelling or shrinking of cells [Sarkadi and Parker, 1991]. More details of the process of cell volume sensing has been reviewed by Chamberlin and Strange [1989] and by Sarkadi and Parker [1991].

Second, after a signal is generated by a volume sensor, this signal will have to be processed in order to quantify the volume change, or to compare it with an internal standard or set point Subsequently, signal transduction mechanisms will be modulated that are species-specific and several studies showed that transport

modulation coincides with phosphorylation of the transport protein [Weinmann *et al.*, 1989; Grinstein *et al.*, 1992; Klein *et al.*, 1993]. Evidence for a separate volume sensing and processing system stems from studies in which an opposite response in transporters involved in RVD or RVI was shown after a cell swelling [Haas and McManus, 1985]. The existence of volume set points, below or above basal cell volume in which range regulatory systems are not activated to avoid simultaneous operation of counteracting transport systems, supports this view [Geck and Heinz, 1986]. The aspect of signal transduction in cell volume regulation will be discussed in the next section.

Third, in the last step transport mechanisms will have to be activated which will redress the volume change towards basal values. An overview of the most important transporters involved in cell volume regulation will be given in a further section. In this thesis the $Na^+/K^+/2Cl$ cotransport, one of the transporters involved in volume recovery after cell shrinkage, has been investigated and will be discussed in more detail. Chapter 4 describes the involvement and regulation of this cotransporter after cell volume perturbation in rabbit proximal tubule cells. In chapter 2 the activity of $Na^+/K^+/2Cl^-$ cotransport in vascular smooth muscle is studied. In chapter 6 expression of $Na^+/K^+/2Cl^-$ cotransport in cultured and differentiated vascular smooth muscle, endothelium, and proximal tubules is investigated.

Signal transduction processes involved in cell volume regulation

Upon cell volume perturbation several signal transduction pathways have been shown to be activated. Besides an important role for $[Ca^{2+}]_i$ [Pierce and Politis, 1990; McCarty and O'Neil, 1992] also IP_3 [Suzuki *et al.*, 1990], calmodulin [Foskett and Spring, 1985; Hoffmann and Simonsen, 1989], cAMP [Matthews *et al.*, 1992], eicosanoids [Lambert *et al.*, 1987], GTP binding proteins [Peña-Rasgado et al, 1994], several protein kinases [Grinstein *et al.*, 1986; Pewitt *et al.*, 1990; Grinstein *et al.*, 1992] and phosphatases [Bianchini *et al.*, 1991; Paulais and Turner; 1992] have been shown to be involved in cell volume regulation. It seems very likely that cell volume is not regulated by only one pathway, but that several pathways and factors are involved that interact with each other. The role of $[Ca^{2+}]_i$ will be discussed in more detail in the next paragraph, since in this thesis changes in $[Ca^{2+}]_i$ in proximal tubule and vascular smooth muscle cells after cell volume perturbation were studied and are described in chapters 2 and 3. The role of $[Ca^{2+}]_i$, protein kinases and phosphatases in the regulation of $Na^+/K^+/2Cl$

cotransport was studied in proximal tubule cells and described in chapter 5. More details about signal transduction pathways that are activated upon cell volume perturbation can be found in a review by Sarkadi and Parker [1991]. Grinstein *et al.* [1992] have reviewed the role of protein kinases and phosphatases in the control of cell volume.

The role of $[Ca^{2+}]_i$ in cell volume regulation

During cell swelling a rapid transient increase in $[Ca^{2+}]_i$ has been observed in several cells which is involved in subsequent RVD [McCarty and O'Neil, 1992]. Cell types in which this rise in $[Ca^{2+}]_i$ has been observed include human lymphocytes [Grinstein *et al.*, 1982], Ehrlich cells [Hoffmann *et al.*, 1984], intestinal epithelial cells [Hazama and Okada, 1988] and rabbit proximal tubule cells [Beck *et al.*, 1991; McCarty and O'Neil, 1991; Breton *et al.*, 1992]. The link between an increase in cell volume and $[Ca^{2+}]_i$ is not clear in most studies, but stretch-activated Ca^{2+} channels appear the most likely transducing elements [Christensen, 1987]. In various cells the rise in $[Ca^{2+}]_i$ is a prerequisite for activation of K^+ and Cl^- channels, which results in efflux of KCl followed by water [McCarty and O'Neil, 1992]. It is not clear whether K^+ and Cl^- conductances are directly activated by Ca^{2+} or indirectly through other Ca^{2+} dependent processes [McCarty and O'Neil, 1992]. In most cells, RVD was dependent on extracellular Ca^{2+} and therefore it was assumed that the source of Ca^{2+} is extracellular [McCarty and O'Neil, 1992]. In cells in which RVD takes place in a Ca^{2+}-free medium it was shown that the increase in $[Ca^{2+}]_i$ is due to release from intracellular stores, as in Ehrlich cells [Hoffmann *et al.*, 1986], rabbit proximal straight tubule [McCarty and O'Neil; 1991] and proximal tubule cells in primary culture as described in chapter 2 of this thesis. Other actions of Ca^{2+} could involve protein phosphorylation [O'Donnell, 1991; Jensen *et al.*, 1993] or an effect on the cytoskeleton [Foskett and Spring, 1985]. A further description of the role of $[Ca^{2+}]_i$ in cell volume regulation has been extensively reviewed elsewhere [Pierce and Politis, 1990; McCarty and O'Neil, 1992].

A role for $[Ca^{2+}]_i$ in RVI is not clear, but several studies reported that RVI is Ca^{2+}-independent [Foskett and Spring, 1985; Grinstein *et al.*, 1982]. Recently Bibby and McCulloch [1994] discovered in human gingival fibroblasts a decline of $[Ca^{2+}]_i$ after hypertonic incubation in 600 mosM medium. In chapter 2 and 3 a similar decrease in $[Ca^{2+}]_i$ after hypertonic incubation is described in rabbit proximal tubules and vascular smooth muscle cells in primary culture.

Transport systems involved in cell volume regulation

In principal, every transport system that gives rise to a net flux of solute in or out of the cell could serve as a cell volume recovery system. However, such a transporter needs to be regulated in an appropriate way. The most frequently described transporters involved in cell volume recovery are listed below and displayed in Fig. 1.

Transport mechanisms most important in RVD are:

1. K^+ efflux functionally coupled to Cl efflux
2. K^+/Cl cotransport
3. K^+/H^+ exchange functionally coupled to Cl /HCO_3 exchange

Additional information about the transport mechanisms involved in RVD can be found in a review by Hoffmann and Simonsen [1989].

Transport mechanisms most important in RVI are:

1. Na^+/K^+/2Cl cotransport
2. Na^+/H^+ exchange functionally coupled to Cl /HCO_3 exchange
3. Na^+/Cl cotransport
4. accumulation of organic compounds

Na^+/K^+/2Cl cotransport and Na^+/H^+ exchange have been reported most frequently in RVI [Eveloff and Warnock, 1987; Hoffmann and Simonsen, 1989; Sarkadi and Parker, 1991]. Two aspects of Na^+/K^+/2Cl cotransport were studied in this thesis. On one hand the possible role of cotransport in RVI of proximal tubule cells was studied and has been described in chapters 4 and 5. On the other hand possible differences in cotransport activity between vascular smooth muscle cells (VSMC) from spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto (WKY) rats were investigated and the results are described in chapter 3 In the following paragraph three techniques are described to measure cell volume which can be used to determine the contribution of Na^+/K^+/2Cl to cell volume recovery. In subsequent paragraphs more detailed information on Na^+/K^+/2Cl cotransport will be given. Besides some general information, both phosphorylation and the recent cloning of the cotransporter will be discussed.

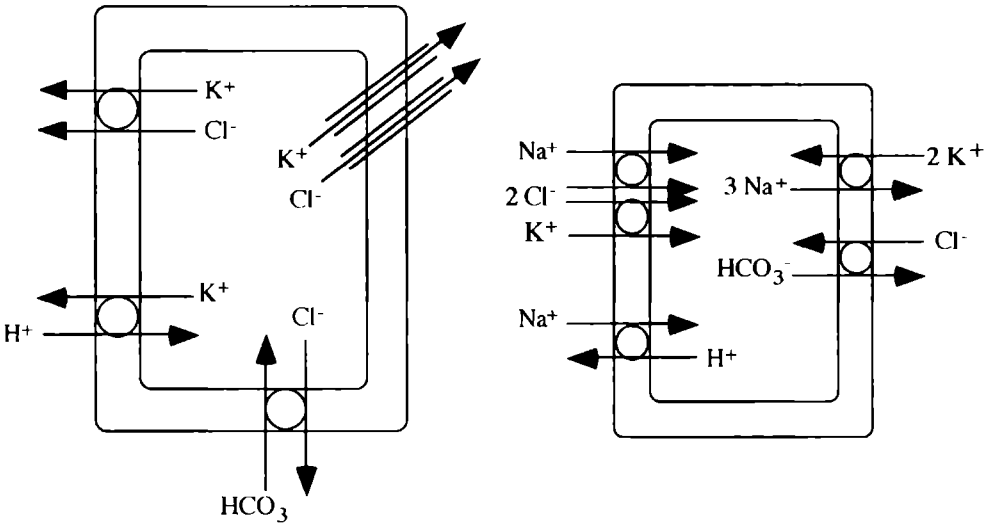


Figure 1
Main transport systems involved in cell volume regulation.

Determining transport activity by measuring changes in cell volume

The contribution of a particular transport system in cell volume regulation can be determined by measuring the course of cell volume recovery both in the absence and in the presence of a specific inhibitor of the transporter. Until now, measuring cell volume has been technically very difficult and has been carried out in isolated renal tubules and cells in suspension [Kimelberg *et al.*, 1992]. Due to the small dimensions and irregular shape it is very complex to measure absolute changes in cell volume in a single attached cell [Tauc *et al.*, 1990]. Therefore, changes in cell volume have been mainly expressed relative to the volume at isotonic conditions. In chapter 4 of this thesis two recently developed techniques to measure cell volume in single attached cells have been evaluated.

By using video imaging the changes in intensity at the ion-insensitive wavelengths of the fluorescent ratio probes fura-2 and BCECF can be used as a measure for changes in cell volume in single attached cells [Tauc *et al.*, 1990; Muallem *et al.*, 1992]. In addition, changes in $[Ca^{2+}]_i$ and pH_i can be measured simultaneously which allows to study the relationship between changes in cell volume and second messengers. Van Driessche *et al.* [1993] developed an automatic cell thickness monitoring system based on the fact that in confluent

epithelial monolayers cells can only increase their cell thickness during cell swelling. The apical and basolateral side of the cell are labelled with fluorescent microbeads. During cell volume perturbation an automatic detection system determines the position of the apical beads in relation to one reference basolateral bead using a video camera and a computer controlled objective. Based on the light distribution of a single fluorescent bead determined from images of successive focal planes the position of the bead can be determined with a accuracy of 0.1 μm.

In addition, the results of these two methods were compared with volume changes measured in a cell suspension using the Coulter counter. This device comprises of a tube with a small 100 μm nozzle through which a suspension of cells is sucked up at such a density that approximately one cell at a time passes the nozzle. The conductance of buffer surrounding the cell is measured in the nozzle and will depend on cell size. An advantage of this method is that it is relatively easy to calibrate this system by using latex beads of known size. Although originally developed to measure volume of blood cells this device can also be used to measure volume of cells that have been isolated from tissue by enzymatic digestion [Roy and Sauvé, 1987; Ehrenfeld *et al* , 1994].

Properties of $Na^+/K^+/2Cl^-$ cotransport

Coupled, electroneutral movement of Na^+, K^+ and Cl occurs nearly in all cell types studied [Haas, 1989, 1994]. Although there has been some dispute about its stoichiometry, it is now agreed that a full turnover results in net translocation of one Na^+, one K^+ and two Cl ions, as originally proposed by Geck *et al.* [1980]. Cotransport can be inhibited by p-sulfamoylbenzoic acid loop diuretics like furosemide and the more specific bumetanide [Palfrey *et al.*, 1980] Rb^+ can replace K^+ stoichiometrically and cotransporter activity is usually determined by measuring the bumetanide-sensitive $^{86}Rb^+$ uptake in the presence of ouabain to inhibit the Na^+/K^+ ATPase [Saier and Boyden, 1984]. In addition, transport of any of the involved ions requires the simultaneous presence of all three ions in the extracellular medium and is inhibited when any one of these ions is removed from the medium [Saier and Boyden, 1984]. The inhibitory site for bumetanide is the second Cl site at the external face of the cell membrane and the loop diuretic binds to the outward facing cotransporter only after Na^+, K^+ and at least one Cl are bound to their respective binding sites [Hegde and Palfrey, 1992]

Electroneutral $Na^+/K^+/2Cl$ cotransport plays a role in renal salt reabsorption in the thick ascending limb of Henle's loop [Eveloff and Calamia, 1986; Sun *et al.*, 1990]. Furthermore, cotransport is involved in salt secretion and has been demonstrated in secretory epithelia like shark rectal gland [Hannafin *et al.*, 1983], salivary gland [Turner *et al.*, 1986] and intestinal [Dharmsathaphorn *et al.*, 1986] and airway [Widdicombe *et al.*, 1983] epithelial cells. In addition, $Na^+/K^+/2Cl$ cotransport serves a significant role in cell volume regulation in both epithelial and non-epithelial cells and has been described in various cell types including vascular smooth muscle cells [Owen, 1984], endothelial cells [O'Donnell, 1989b], fibroblasts [Owen and Prastein, 1985], osteoblasts [Whisenant *et al.*, 1991], cardiac cells [Frelin *et al.*, 1986], astrocytes [Kimelberg and Frangakis, 1986], Ehrlich ascites tumor cells [Hoffmann *et al.*, 1983], lymphocytes [Feldman, 1992] and erythrocytes [Haas *et al.*, 1982].

There are also indications that $Na^+/K^+/2Cl$ cotransport is involved in the regulation of cell volume in cells during growth and differentiation. In mouse erythroleukemia cells, differentiation results in a simultaneous decline in cell volume and a decrease in cotransport activity which is responsible for the major salt influx in undifferentiated cells [Delpire and Gullans, 1994]. In addition, $Na^+/K^+/2Cl$ cotransport has also been reported to play a role in regulation of cell proliferation [Panet *et al.*, 1994].

In a variety of tissues $Na^+/K^+/2Cl$ cotransport activity is affected by hormones and second messengers that activate cyclic-nucleotide dependent protein kinases and protein kinase C [Geck and Heinz, 1986; Haas, 1989, O'Donnell, 1991]. $Na^+/K^+/2Cl$ cotransport activity, therefore, is modulated by many factors [Haas, 1989] including beta-adrenergic agents, vasopressin, atrial natriuretic factor, epidermal growth factor, insulin, and thrombin Whether these agents have a stimulatory or inhibitory effect is tissue dependent [Haas, 1989].

Early evidence for the fact that phosphorylation is a prerequisite for activation of $Na^+/K^+/2Cl$ cotransporter stems from observations that ATP depletion inhibited cotransport activity [Altamirano, 1988] In various tissues cotransport can be activated or inactivated by cAMP, phorbol esters, and cytosolic-free calcium [Haas, 1989]. Compelling evidence for phosphorylation of the $Na^+/K^+/2Cl$ cotransport was reported by Pewitt *et al* [1990], as they showed that in duck red cells kinase inhibitors like 252a and H-9 block the activation of $Na^+/K^+/2Cl$ cotransport. Okadaic acid, a protein serine/threonine phosphatase

inhibitor [Suganuma *et al*., 1988], stimulated $Na^+/K^+/2Cl^-$ cotransport and bumetanide binding and increased the level of phosphorylation of many membrane proteins. Recently, Lytle *et al.* [1992] have shown that a 200 kD bumetanide-binding membrane protein from shark rectal gland is phosphorylated in response to activation by forskolin and also by changes in cell volume. In chapter 5 of this thesis the role of several protein kinases, second messengers and phosphatases in regulating $Na^+/K^+/2Cl^-$ cotransport activity in primary cultures of proximal tubule cells have been studied

Molecular Biology of $Na^+/K^+/2Cl^-$ cotransport

Until recently, very little was known about the structure and molecular properties of the $Na^+/K^+/2Cl^-$ cotransport system. Photoaffinity labelling studies using a photosensitive bumetanide analog showed labelling of ~ 150 kD proteins in dog kidney, mouse kidney, and duck red blood cell membranes [Haas, 1994] and a glycosylated ~195 kD protein from shark rectal gland [Lytle *et al*., 1992] Monoclonal antibodies to the native shark cotransporter were used to isolate a cDNA encoding a $Na^+/K^+/2Cl^-$ cotransporter from a shark rectal gland cDNA expression library In the beginning of 1994 this resulted in a publication by Xu *et al.* [1994] describing the molecular cloning and functional expression of the $Na^+/K^+/2Cl^-$ cotransporter from shark rectal gland (NKCC1) in the human embryonic kidney cell line HEK-293 Northern blot analysis identified a 7.4 kb transcript in rectal gland and most other tissues whereas a 5.2 kb transcript was restricted to shark kidney. The cDNA sequence predicts a protein of 1191 amino acids with 12 putative transmembrane domains and a molecular weight of about 130 kD (Fig. 2A). The sequence predicted two phosphorylation sites for PKC (Thr-189 and Thr-1114) that have earlier been shown to be phosphorylated in the rectal gland during activation of the cotransporter [Lytle and Forbush, 1992, Xu *et al.*, 1994]

Only 4 months later Gamba *et al.* [1994] described the isolation of a ~ 4.6 kb $Na^+/K^+/2Cl^-$ cotransport transcript rBSC1 (rat bumetanide sensitive cotransporter) by screening a cDNA library from the inner stripe of rat kidney outer medulla using probes based on the earlier cloned thiazide-sensitive Na^+/Cl^- cotransporter of flounder urinary bladder (fITSC) [Gamba *et al.*, 1993] The 3,285 bp coding segment of the rBSC1 cDNA predicts a protein of 1095 amino acids and a molecular weight of approximately 120 kD as determined by *in vitro* translation Its functional activity was tested in *Xenopus* oocytes where it was found to

express a bumetanide-sensitive $^{86}Rb^+$ uptake which was 100-fold higher than in water-injected oocytes. In the kidney, the transcript was localized in superficial cortex, cortex, outer medulla and inner medulla. These findings indicate that rBSC1 cDNA encodes the bumetanide-sensitive $Na^+/K^+/2Cl$ cotransporter expressed on apical membranes of the thick ascending limb of Henle (TAL) [Gamba *et al.*, 1994].

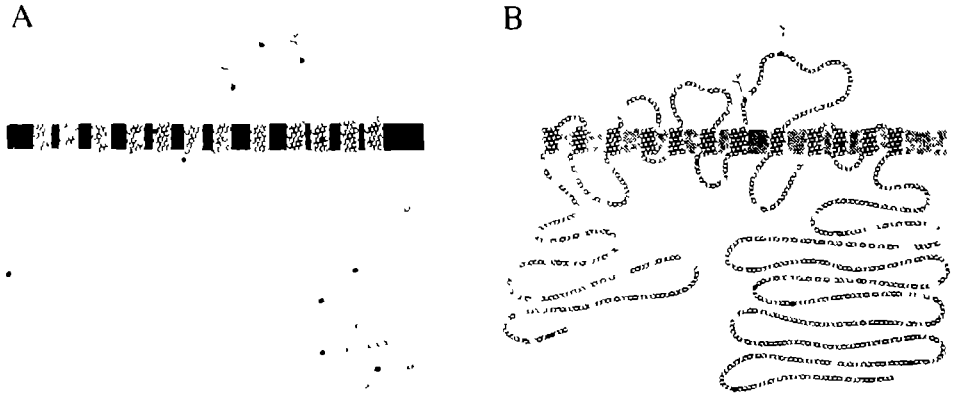


Figure 2

A Proposed model of the shark $Na^+/K^+/2Cl$ cotransporter NKCC1 as described by Xu *et al.* [1994]. **B.** Proposed model of the rabbit renal $Na^+/K^+/2Cl$ cotransporter NKCC2 as described by Payne and Forbush [1994].

Payne and Forbush [1994] have used cDNA probes derived from the secretory $Na^+/K^+/2Cl$ cotransport (NKCC1) to screen cortical and medullary rabbit kidney cDNA libraries resulting in the cloning of an isoform named NKCC2. The cDNA predicts a protein consisting of 1099 amino acids, 12 transmembrane domains and a molecular weight of ~ 121 kD (Fig. 2B). NKCC1 and NKCC2 showed 61% identity with the highest degree in the predicted transmembrane helices. With an antisense cRNA probe which encoded a conserved region of NKCC2, a band at 5.1 kb was found to be expressed at very high level in rabbit kidney, but not in other tissues. With the same probe a 7.4 kb transcript was found in shark rectal gland confirming the close identity of NKCC2 to NKCC1 as was found earlier by Xu et al. [1994]. These findings suggest that NKCC2 encodes the absorptive form of the cotransporter found in

renal tubule Three alternatively spliced variants of the NKCC2 cDNA were identified named A, B and F, which differed only in a 96 bp region that encodes a putative transmembrane segment 2 and a part of the adjacent intracellular loop Variant A was distributed in both cortex and medulla, variant B was exclusively found in the cortex and variant F was found to be restricted to the medulla The localization of variant F to the medulla and its concentration suggests that this variant encodes the putative $Na^+/K^+/2Cl$ cotransporter from medullary TAL The observations also suggest that $Na^+/K^+/2Cl$ cotransporter is widely distributed in the mammalian kidney and is not restricted to the TAL segment.

Recently, Delpire *et al.* [1994] cloned a putative basolateral $Na^+/K^+/2Cl$ cotransporter from mouse inner medullary collecting duct (mIMCD-3) cells based on its high degree of homology with flounder [Gamba *et al*., 1993] and rat thiazide-sensitive Na^+/Cl cotransporters (flTSC and rTSC, respectively) and the rat apical bumetanide-sensitive $Na^+/K^+/2Cl$ cotransporter (rBSC) [Gamba *et al*., 1994]. The 4.7 kb mBSC2 cDNA encodes a protein consisting of 1205 amino acids with a molecular weight of ~ 130 kD. In accordance with the other cotransporter proteins, 12 potential transmembrane domains were predicted from hydropathy analysis. Northern blot analysis revealed the presence of a major 6.5 kb transcript and two less abundant transcripts at 3.9 and 4.7 kb. The 6.5 kb transcript was found to represent an extension of the 3'-untranslated sequence with an alternative polyadenylation site. mBSC2 was found to be most related to the shark NKCC1 cDNA indicating that mBSC2 encodes a putative basolateral $Na^+/K^+/2Cl$ cotransporter participating in salt and/or fluid secretion [Delpire *et al*., 1994]. Additional evidence stems from the fact that expression of mBSC2 mRNA was found in several tissues in contrast to rBSC1 [Gamba *et al*., 1994] and NKCC2 mRNA [Payne and Forbush, 1994] The latter two are both restricted to the kidney, located in the apical membrane and are involved in salt reabsorption. Delpire *et al.* [1994] suggest that in non-polarized cells the "basolateral" isoform, which has been found to be more sensitive to cell shrinkage [Hebert and Sun, 1988], likely plays a role in cell volume regulation In chapter 6 of this thesis a 1200 bp PCR fragment of mBSC2 was used to study the differences in $Na^+/K^+/2Cl$ cotransport RNA expression in cells in primary culture and in the native tissues of rabbit proximal tubule, rat vascular smooth muscle and porcine aortic endothelium

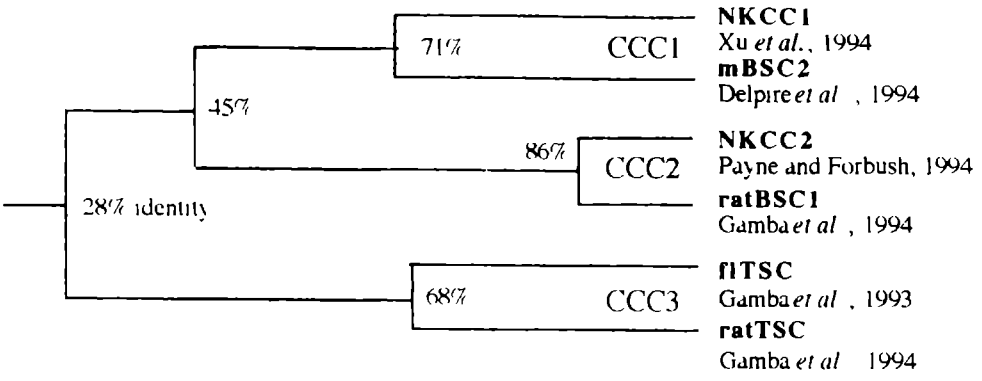


Figure 3
Homology tree of cation-chloride cotransporter (CCC) family cDNAs with additional properties of the different cotransport members The percentage of identity between the nucelotide sequence of the coding region of the different proteins was calculated by Delpire *et al* [1994] using the unweighted pair group method with arithmetic mean algorithm The estimated genetic distance between the cDNAs is proportional to the length of the lines (Modified from Delpire *et al* [1994])

Haas [1994] has suggested a cation-Cl cotransporter family (CCC) existing of three groups in which the different cotransporters that have been cloned until now can be classified. The bumetanide-sensitive and thiazide-sensitive cotransporters share a respectable amino acid sequence identity as indicated in Fig. 3. The greatest degree of sequence divergence among the different cotransporters is in the hydrophilic NH_2- and COOH terminal domains and the predicted extracellular loops which are probably sites for loop diuretic and azide binding.

The CCC family not only comprises of different isoforms of the $Na^+/K^+/2Cl$ cotransporter but also cotransporters which differ in ion requirement and transport stoichiometry and have different inhibitor sensitivities. Other members of the CCC family could be the bumetanide-sensitive Na^+/Cl cotransporters [Eveloff and Calamia, 1986; Sun *et al.*, 1990], and several K^+/Cl cotransporters [Reuss, 1983; Lauf *et al.*, 1992] The identification of additional members of the family will help to clarify previous discussions on the relation between different cotransport mechanisms.

Aim of this thesis

In this thesis two different cell types, rabbit proximal tubules and vascular smooth muscle from rat aorta, have been studied which under physiological conditions are not exposed to anisosmotic conditions. However, in both cell types changes in intracellular osmolarity could introduce changes in cell volume which activate volume regulatory volume mechanisms In the proximal tubule ~ 70% of the Na^+ and water of the glomerular filtrate is reabsorbed by active Na^+ transport mechanisms [Berne and Levy, 1993]. For an individual proximal tubule cell this amounts to a volume of water one to four times that of the cell volume which is reabsorbed every minute [Lohr and Grantham, 1986]. Volume regulation will, therefore, be of great importance when solute in- or efflux are suddenly altered. In vascular smooth muscle cells (VSMC) isolated from rat aorta of spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto (WKY) rats differences in $Na^+/K^+/2Cl$ cotransport have been demonstrated [Tokushige *et al.*, 1986; Kuriyama *et al.*, 1988; Orlov *et al* , 1992]. Apart from a consequence on cell volume, cotransport will influence the Na^+ gradient and might, via the $3Na^+/Ca^{2+}$ exchanger, be involved in the disturbed Ca^{2+} homeostasis that has been reported in VSMC of SHR [Bohr and Webb, 1988, Sharma and Bhalla, 1988].

In chapter 2 the changes in $[Ca^{2+}]_i$ and pH_i were studied in cultured proximal tubule cells after cell volume perturbation by anisosmotic media. Chapter 3 presents the differences in osmolarity-induced changes in $[Ca^{2+}]_i$ between VSMC isolated from SHR and WKY. In addition, the activity of $Na^+/K^+/2Cl$ cotransport was studied in both cell types. In chapter 4 three different methods to measure changes in cell volume were compared in a monolayer of rabbit proximal tubule cells in primary culture. The presence of $Na^+/K^+/2Cl$ cotransport in cultured rabbit proximal tubule cells was studied in chapter 5 and the role of several signal transduction pathways in activation of cotransport was determined The difference in $Na^+/K^+/2Cl$ cotransport RNA expression levels between fully differentiated and cultured vascular smooth muscle, endothelium and proximal tubule was studied in chapter 6. Finally, the various results from the separate chapters have been summarized and discussed in chapter 7

CHAPTER 2

Effects of osmotic perturbation on intracellular Ca^{2+} and H^+ concentration in rabbit proximal tubular cells in primary culture

N.J.H. Raat, C.H. van Os and R.J.M. Bindels

Am. J. Physiol., 1995 (in press)

SUMMARY

The effects of anisosmotic media on intracellular Ca^{2+} and H^+ concentrations, $[Ca^{2+}]_i$ and pH_i, were studied to investigate whether these changes play a role in epithelial cell volume regulation. $[Ca^{2+}]_i$ and pH_i were measured in rabbit proximal tubular cells in primary culture using the fluorescent ratio probes fura-2 and BCECF. Changing medium osmolarity from 300 to 150 mosM resulted in a rapid transient increase in fura-2 ratio from 0.89 ± 0.02 to 1.15 ± 0.03 which lasted for several minutes and returned to base line within 10 min. The source of Ca^{2+} was extracellular as well as intracellular. Simultaneous with this increase in $[Ca^{2+}]_i$, cells slowly acidified from pH_i of 7.51 ± 0.03 to 6.86 ± 0.02. This osmotic swelling induced acidification could not be explained by a decrease in the rate of Na^+/H^+ exchange or increase in the rate of Cl^-/HCO_3^- exchange. Subsequently increasing medium osmolarity from 150 to 500 mosM decreased the fura-2 ratio below the initial level observed in isotonic media while pH_i increased from 6.96 ± 0.02 to 7.37 ± 0.03. This decrease in $[Ca^{2+}]_i$ was due to inhibition of Ca^{2+} influx and to an increase in Ca^{2+} efflux. The osmotic shrinkage induced alkalinization was slightly inhibited by ethyl-isopropyl amiloride, indicative of activation of Na^+/H^+ exchange.

To test whether an increase in $[Ca^{2+}]_i$ causes a decrease in pH_i or vice versa, pH_i and $[Ca^{2+}]_i$ were manipulated at isotonic conditions. Surprisingly, a decrease in $[Ca^{2+}]_i$ was accompanied by a decrease in pH_i and an increase in pH_i resulted in an increase in $[Ca^{2+}]_i$ in the absence of osmotic perturbation. In conclusion, changes in $[Ca^{2+}]_i$ and pH_i resulting from osmotic perturbation of proximal tubular cells in primary culture appear to be independent phenomena. This study suggests that both $[Ca^{2+}]_i$ and pH_i play a role as second messengers in cell volume regulation.

INTRODUCTION

Most cells exposed to anisosmotic solutions respond by activating volume recovery systems to prevent cell damage by either cell swelling or cell shrinkage [Eveloff and Warnock, 1987; Hoffmann and Simonsen, 1989; Lewis and Donaldson, 1990; Sarkadi and Parker, 1991]. In addition, in epithelial tissues like proximal tubule (PT) which are not subjected to large changes in extracellular osmolarity volume recovery systems will be needed when the rate of transcellular solute transport is suddenly altered. The rate of water and ion transport across the proximal tubular cell is the highest observed among epithelial tissues, and up to 4 times its intracellular volume can be transported each minute by an individual PT cell [Lohr and Grantham, 1986]. Changes in the availability of transportable solutes or the presence of transport modulating hormones will affect transport rate and can lead to imbalance of influx and efflux followed by cell volume perturbation.

An increase in intracellular calcium concentration ($[Ca^{2+}]_i$) could play a role in the process of regulatory volume decrease (RVD) observed after osmotic cell swelling [McCarty and O'Neil, 1992]. Such an increase in $[Ca^{2+}]_i$ could activate K^+ and Cl^- channels which results in KCl efflux followed by water and restoration of cell volume. In PT cells of the rabbit an increase in $[Ca^{2+}]_i$ after cell swelling has been reported [Suzuki *et al.*, 1990; Beck *et al.*, 1991; McCarty and O'Neil, 1991; McCarty and O'Neil, 1992]. In contrast to the multitude of studies dealing with osmotic swelling, few reports have studied $[Ca^{2+}]_i$ after cell shrinkage or during regulatory volume increase (RVI). Since changes in $[Ca^{2+}]_i$ and intracellular pH (pH_i) are often interrelated we studied both $[Ca^{2+}]_i$ and pH_i during osmotic perturbation of PT cells in primary culture.

MATERIALS AND METHODS

Materials

All chemicals were obtained from Merck (Darmstadt, Germany) unless otherwise specified Bumetanide was obtained from Leo Pharmaceutical Products (Ballerup, Denmark) Fetal calf serum was purchased from Sera Lab (Sussex, UK), HEPES and TRIS from Research Organics (Cleveland, OH, USA) Gentamycin was obtained from Schering Corporation (Kenilworth, NJ, USA) Fura 2 AM, BCECF AM, BAPTA AM and ethyl-isopropylamiloride (EIPA) from Molecular Probes (Eugene, OR, USA) The disodium salt of 4,4' diisothiocyanatostilbene 2,2' disulfonic acid (DIDS) was purchased from Sigma (St Louis, MO, USA)

Solutions and media

The isotonic (300 mosM) medium was a Krebs Henseleit buffer (KHB) which contained (in mM) 110 NaCl, 5 KCl, 2 NaH_2PO_4, 1 2 $MgSO_4$, 10 sodium acetate, 4 L lactate, 10 D-glucose, 1 L-alanine, 20 HEPES and 1 $CaCl_2$, calibrated with TRIS to pH 7 4 Hypotonic medium (150 mosM) was similar to this solution except that NaCl concentration was reduced to 50 mM Hypertonic medium (500 mosM) was isotonic KHB to which 200 mM mannitol had been added Nominally Ca^{2+} free medium contained no $CaCl_2$ and 0 1 mM $LaCl_3$ was used to inhibit residual Ca^{2+} influx Chloride free medium was KHB in which NaCl and KCl had been replaced by Na^+ and K^+ gluconate NH_4Cl medium was KHB in which 30 mM NaCl was replaced by NH_4Cl The osmolarity of the solutions was checked with an osmometer (Osmette A, Precision Systems, Sudbury, MA, USA) and adjusted to the desired value with mannitol K_1 medium consisted of a 1 1 mixture of Dulbecco's modified Eagle's medium (Imperial, Hampshire, UK) and Ham's F12 medium (Gibco, Paisley, UK), supplemented with gentamycin (10 μg/ml), $NaHCO_3$ (25 mM), glutamine (14 mM) 0 5% (vol/vol) 100 x non essential amino acids (Gibco, Paisley, UK), insulin (5 μg/ml), transferrin (5μg/ml), hydrocortisone (50 nM), prostaglandin E_1 (70 ng/ml), triiodothyronine (5 pM), Na_2SeO_3 (50 nM) and the pH was set at 7 4

Primary culture of rabbit kidney proximal tubule cells

Rabbit kidney proximal tubule (PT) cells were isolated and subsequently cultured as described previously [Rose *et al*, 1993] Briefly, PT cells were immunodissected from rabbit kidney with monoclonal antibodies 85C8 and 101E12 Cells were seeded at a density of 2 10^5 cells/cm^2 on round 22 mm coverslips which were coated with rat tail collagen (~40 $\mu g/cm^2$) and cultured to confluency in K_1 medium in an atmosphere equilibrated with 5% CO_2 95% air at 37 °C During the first 24 hours in culture, K_1 was supplemented with 5% (vol/vol) fetal calf serum Medium was changed every other day and the day before experiments were performed PT cells were used 6 days after seeding

Measurement of $[Ca^{2+}]_i$ and pH_i

PT cells cultured on 22 mm round coverslips were loaded with fura 2 AM for 1 hr in KHB medium (300 mosM) containing 5 μM fura 2-AM, 0 02% (wt/vol) pluronic F127 and 3 mM probenecid in a shaking water bath at 37 °C After loading, the coverslip with cells was

transferred to a thermostatic "Leiden" chamber [Ince *et al.*, 1983]. The volume of the chamber was reduced to 200 μl by a perspex insert. For experiments in which $[Ca^{2+}]_i$ and pH_i were measured simultaneously, cells were subsequently loaded in about 200 μl KHB medium containing 1 μM BCECF-AM until fluorescence intensity equalled that of fura-2 (usually within 5 min). The chamber was mounted on the stage of a Nikon Diaphot inverted microscope equipped with a 40x quartz oil immersion objective to monitor single PT cells. Fluorescence was captured by a low-level charge-coupled device (CCD) camera and further processed by TARDIS software on the MagiCal system (Applied Imaging, Tyne and Wear, UK). The MagiCal system has been extensively described by Neylon *et al.* [1990]. Cells were superfused with KHB at 37 °C at a rate of 1.5 ml/min. Probenecid (0.3 mM) was present in the media to reduce dye leakage from the cells. Before experiments, cells were pre-incubated for 10 min in isotonic KHB. pH_i was calculated from the 490/440 nm ratio ($R_{490\,440}$) of BCECF after calibration in a medium containing (in mM): 112 K^+ gluconate, 28 KCl, 10 NaCl, 1 $MgCl_2$, 0.01 $CaCl_2$, 5 HEPES, 10 D-glucose, 20 mannitol and 0.01 nigericin. In this buffer, a 4-point calibration series between pH 6.0 and 7.8 was prepared by adjusting pH with TRIS. Calibration in 8 single cells per coverslip from 3 different preparations were pooled and a mean calibration curve was calculated. The data points were fitted by linear regression to obtain a correlation between $R_{490\,440}$ and pH_i. After an NH_4Cl pulse the rate of increase in pH_i was determined as described by Abrahamse *et al.* [1992]. The data was fitted to the following exponential form: $R_t = R_{st} - (R_{st} - R_{init}).e^{kt}$. R_t is the $R_{490\,440}$ at time t, R_{st} is the $R_{490\,440}$ at steady state, R_{init} is the $R_{490\,440}$ at time t= 0, and k is the rate constant for pH_i recovery. The rate of the change in $R_{490\,440}$ (dR/dt) at pH_i 6.8 ($R_{6,8}$) was calculated as described by Simchowitz and Roos [1985] : dR/dt = k.(Rst - $R_{6,8}$). The rate of pH_i recovery (dpH_i/dt) was calculated from dR/dt by using the calibration curve. Calibration of fura-2 ratios in situ were severely hindered by the effects of ionomycin and EGTA on cultured PT cells. In most attempts addition of ionomycin or EGTA resulted in detachment of cells from the coverslip or lysis of cells, observations which have been previously encountered and described by Rose *et al.* [1993]. Therefore, throughout the paper $[Ca^{2+}]_i$ levels are represented as fura-2 ratios.

Protocol for study of changes in $[Ca^{2+}]_i$ and pH_i after cell swelling and shrinkage

After loading with fluorescent probes the cells were pre-incubated for 10 min in 300 mosM KHB at 37° C and subsequently $[Ca^{2+}]_i$ or pH_i were measured. The first 5 min cells were superfused with 300 mosM medium to obtain the initial level of fluorescence. To induce cell swelling, cells were superfused for 10 min with a 150 mosM medium. After this period cells were superfused for 10 min with a 500 mosM medium. When the effect of EIPA on pH_i was tested during incubation in 500 mosM, EIPA (10 μM) was already added 5 min before incubation in hypertonic medium.

Statistics

Measurement were performed on cells derived from two or three different preparations. From each preparation 16 cells were measured Statistical significance was determined by one way analysis of variance (ANOVA) or a paired t-test. Data is presented as the mean ± SE.

RESULTS

Effect of cell swelling on $[Ca^{2+}]_i$ and pH_i

Exposure of primary cultured PT cells to hypotonic medium resulted in a fast, but transient, increase in fura-2 ratio within the first minute (Fig. 1 and Table 1) Within 10 min, the fura 2 ratio had decreased below the initial level to 0 74 ± 0.01 A heterogeneity in the fura-2 ratio response was observed, even among cells measured on the same coverslip (Fig. 1A, B and C)

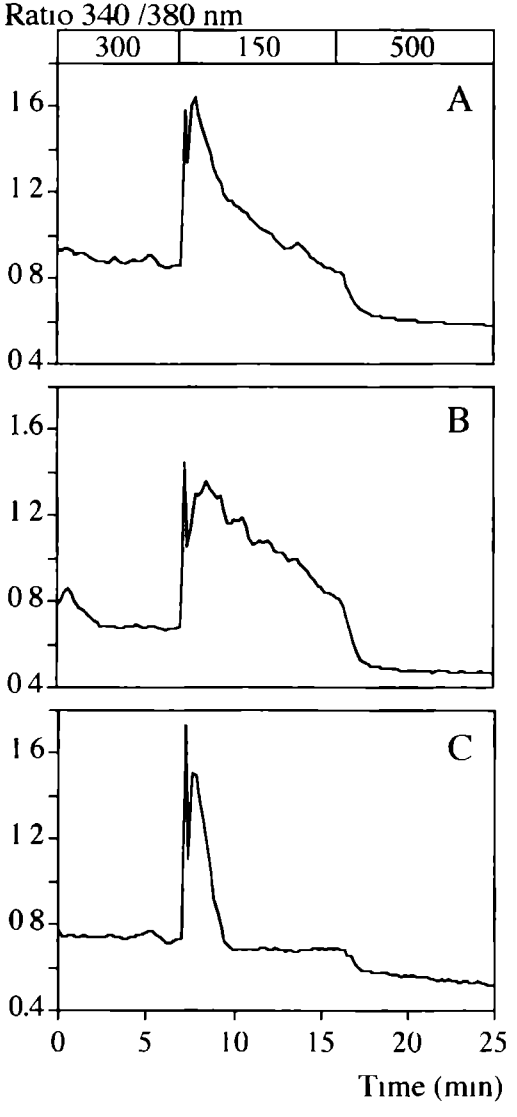


Figure 1
Heterogeneity in cellular Ca^{2+} response in three single primary cultured rabbit PT cells in the same confluent monolayer during incubation in hypotonic (150 mosM) and hypertonic (500 mosM) media

Table 1. Change in $[Ca^{2+}]_i$ during cell swelling and subsequent cell shrinkage.

	FURA-2 RATIO				
	hypotonicity		hypertonicity		
Condition	before	after	before	after	n
control	0.89 ± 0.02	1.15 ± 0.03 *	0.74 ± 0.01	0.65 ± 0.01 *	96
Ca^{2+} free	0.70 ± 0.01	0.92 ± 0.03 *	0.69 ± 0.02	0.58 ± 0.01 *	48
thapsigargin	0.85 ± 0.02	0.95 ± 0.04 *	0.73 ± 0.02	0.64 ± 0.02 *	32
thapsigargin + Ca^{2+} free	1.11 ± 0.05	0.66 ± 0.03 *	0.67 ± 0.03	0.58 ± 0.02 *	32

Fura-2 ratio was measured during cell swelling in 150 mosM medium and subsequent cell shrinkage in 500 mosM medium. Initial values were compared with values directly after hypo- or hypertonicity in which nominally Ca^{2+} free medium (+ 0.1 mM $LaCl_3$), 1 μM thapsigargin, or both together were present.Values are mean ± SE of n different cells from 2 or 3 separate isolations. * significantly different from value before osmotic perturbation, $P < 0.05$.

The various responses in fura-2 ratio after a hypotonic shock could be divided in three groups, differing in their time course of recovery. As based on this arbitrary classification, the response shown in Fig. 1A was observed in ~46% of all responses. The other responses where observed in similar frequencies of ~26 and ~24% for response 1B and 1C, respectively, while ~4% of the cells did not respond with an increase in $[Ca^{2+}]_i$.Before osmotic perturbation, pH_i was 7.51 ± 0.03 Table 2) and during hypotonicity pH_i decreased slowly to 6.96 ± 0.02 within 10 min (Fig. 2). In contrast to the $[Ca^{2+}]i$, pH_i responded rather uniform.

The transient increase in $[Ca^{2+}]_i$ upon exposure to hypotonic medium could arise from Ca^{2+} influx, from release of intracellular Ca^{2+}, or from both. As shown in Fig. 3A, PT cells preincubated in nominally Ca^{2+} free medium for 3 min, still responded with a transient increase in $[Ca^{2+}]_i$ (Table 1) upon changing to a hypotonic nominally Ca^{2+} free medium. Depletion of intracellular Ca^{2+} stores by preincubation with thapsigargin before osmotic perturbation still resulted in an increase in fura-2 ratio compared with control values (Table 1, Fig. 3B). Depletion of intracellular Ca^{2+} stores by thapsigargin and subsequent exposure to a nominally Ca^{2+} free hypotonic medium completely abolished the increase in fura-2 ratio (Table 1, Fig. 3C). In this situation $[Ca^{2+}]_i$ decreased significantly below the initial value.

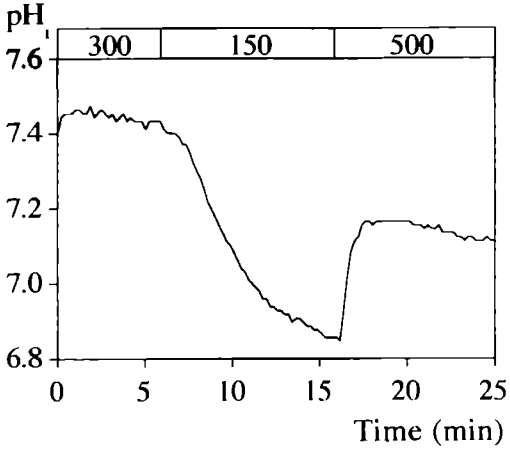
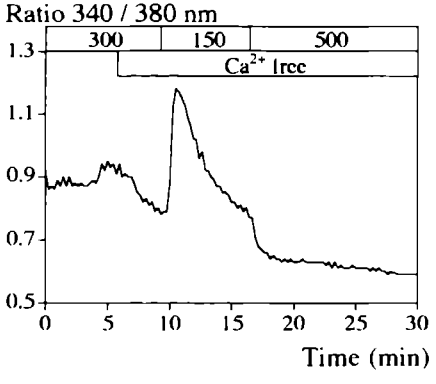


Figure 2
Typical response in pH_i in one single rabbit PT cell in a confluent monolayer after hypotonic (150 mosM) and subsequent hypertonic incubation (500 mosM). A homogenous response was observed both in cells measured on one coverslip as well as in cells from different isolations.



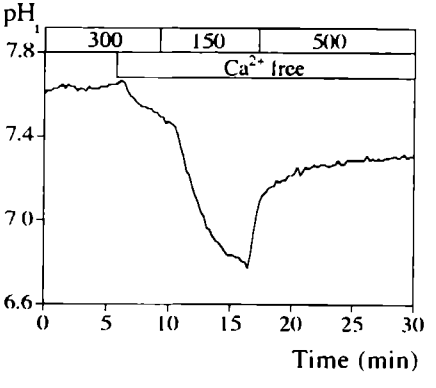


Figure 3A
Typical response in simultaneously measured fura-2 ratio and pH_i in a single rabbit PT cell in a confluent monolayer during nominal Ca^{2+} free hypo- and hypertonic incubation. During pre incubation in nominally Ca^{2+} free isotonic medium a decrease in fura-2 ratio is observed, but an increase in fura-2 ratio is still present when cells are incubated in nominally Ca^{2+} free hypotonic (150 mosM) medium. The fura-2 ratio decreases when cells are subsequently placed in hypertonic (500 mosM) medium.

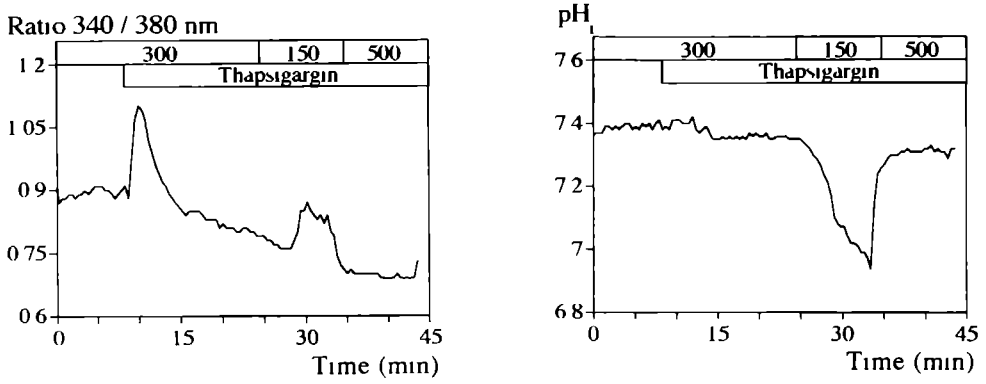


Figure 3B

Typical response in simultaneously measured fura 2 ratio and pH_i in a single rabbit PT cell in a confluent monolayer during hypo- and hypertonic incubation after depletion of intracellular Ca^{2+} stores by 1 μM thapsigargin Addition of thapsigargin results in an increase in fura-2 ratio After hypotonic (150 mosM) incubation the rise in fura 2 ratio is significantly lowered compared to control The fura-2 ratio decreases further when cells are subsequently placed in hypertonic (500 mosM) medium

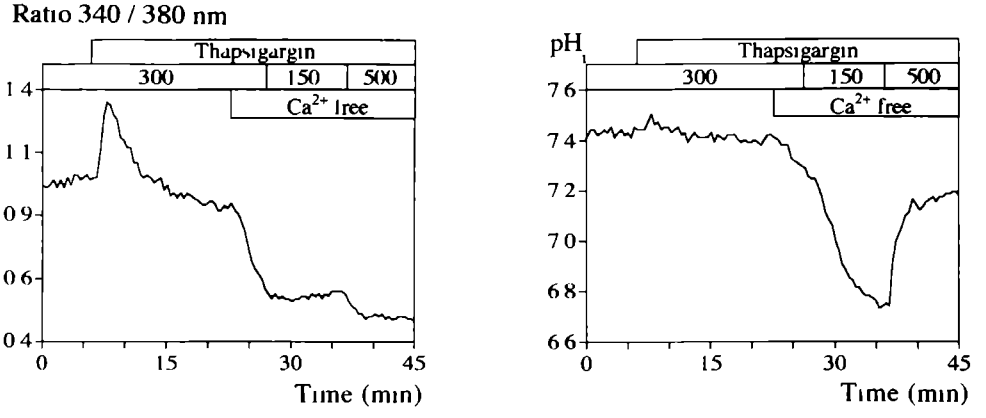


Figure 3C

Typical response in simultaneously measured fura-2 ratio and pH_i in a single rabbit PT cell in a confluent monolayer during hypo- and hypertonic incubation after initial depletion of intracellular Ca^{2+} stores by 1 μM thapsigargin and subsequent incubation in nominally Ca^{2+} free hypotonic (150 mosM) medium The increase in fura 2 ratio is completely abolished. The fura 2 ratio further declines when cells are subsequently placed in hypertonic (500 mosM) nominally Ca^{2+} free medium

Upon cell swelling an acidification of the cytosol was observed. Due to the low extracellular [Na^+] in the hypotonic medium a reversed operation of the Na^+/H^+ exchanger could be responsible for this acidification. However, cell acidification after hypotonic swelling was not significantly reduced by addition of EIPA (Table 2). Other processes responsible for acidification could be efflux of H^+ from intracellular organelles, or activation of Cl^-/HCO_3^- exchange [Muallem *et al.*, 1992]. Although HCO_3^- was not present in our incubation medium it is feasible that OH^- efflux via the anion exchanger is the cause of cellular acidification. In Cl^- free media cell swelling is still accompanied by acidification (Table 2). In addition, the presence of 0.1 mM DIDS during the hypotonic shock was also without effect (Table 2). The possibility of conductive efflux of OH^-, as described by Muallem *et al.* [1992] in osteosarcoma cells, was excluded by incubation in a high K^+ (50 mM) hypotonic medium. High K^+ will depolarize PT cells and thereby reduce the driving force for conductive OH^- efflux, but acidification was still observed (data not shown).

Table 2. Change in pH_i during cell swelling and subsequent cell shrinkage

	pH_i			
	hypotonicity		hypertonicity	
Condition	before	after		n
control	7 55 ± 0 03	6 99 ± 0 02 *	7 41 ± 0 03 *	80
Cl free	7 74 ± 0 03	7 02 ± 0 03 *	7 47 ± 0 04 *	48
+ EIPA	7 72 ± 0 05	7 03 ± 0 04 *	7 49 ± 0 04 *	32
+ DIDS	7 63 ± 0 02	7 01 + 0 02 *	7 48 ± 0 05 *	48

pH_i was measured using BCECF during cell swelling in 150 mosM medium and subsequent cell shrinkage in 500 mosM medium Initial values were compared with values after 10 min in Cl-free medium, or in medium in which 10 μM EIPA, or 0 1 mM DIDS were present Values are mean ± SE of n different cells from 2 or 3 separate isolations * significantly different from value before osmotic perturbation, $P < 0.05$

Effects of cell shrinkage on $[Ca^{2+}]_i$ and pH_i

Changing the osmolarity from 300 to 500 mosM resulted in a decrease in fura-2 ratio from a basal level of 0.82 ± 0 04 to 0.67 ± 0.03 (Fig 4A) During this manoeuvre pH_i increased from 7.69 ± 0 03 to 7.85 ± 0 04 (Fig 4B) Superfusion of PT cells with 500 mosM after superfusion with 150 mosM for 10 min also resulted in a decrease in the fura-2 ratio and an increase in pH_i (Table 2, Fig. 2)

The observed decline in $[Ca^{2+}]_i$ upon cell shrinkage could be brought about by: I. inhibition of Ca^{2+} influx not balanced by a decrease in Ca^{2+} efflux, II. increased Ca^{2+} sequestration by intracellular stores, or by III. increased Ca^{2+} efflux. During incubation in nominally Ca^{2+} free medium the decline in $[Ca^{2+}]_i$ upon cell shrinkage is still present (Fig. 3A, Table 1). The decrease in $[Ca^{2+}]_i$ is also observed in the presence of thapsigargin (Fig. 3B) and in the absence of Ca^{2+} and presence of thapsigargin (Fig. 3C). Since no specific inhibitors of the plasma membrane Ca^{2+}- ATPase are available at present, experiments to directly test the involvement of this Ca^{2+} pump can not be experimentally evaluated.

Whether Na^+/H^+ exchange is responsible for the alkalinization upon cell shrinkage was tested using the inhibitor EIPA. Changing the medium osmolarity from 300 to 500 mosM medium in the presence of EIPA did not reduce alkalinization. However, pre-swelling in 150 mosM followed by 500 mosM in the presence of EIPA resulted in a lower pH_i value than in the absence of EIPA (Table 2). When pH_i was acidified by an NH_4Cl pulse the recovery of pH_i (dpH_i/dt) was 0.22 ± 0.05 pH units/min (means ± SE, n=16) which decreased in the presence of EIPA to 0.07 ± 0.01 pH units/min (means ± SE, n=12).

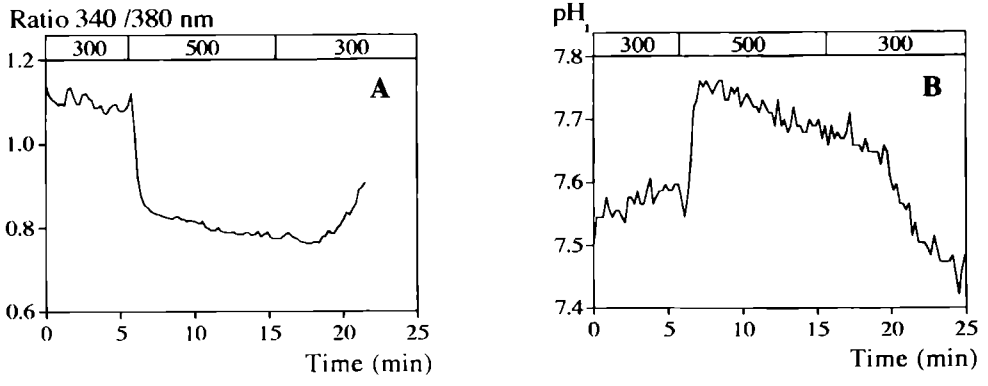


Figure 4

A. Decrease in fura-2 ratio after hypertonic (500 mosM) and subsequent isotonic incubation (300 mosM) in one cell in a confluent monolayer of rabbit PT cells.

B. Increase in pH_i after hypertonic (500 mosM) and subsequent isotonic incubation (300 mosM) in one cell in a confluent monolayer of rabbit PT cells.

Interdependence of $[Ca^{2+}]_i$ and pH_i

During cell swelling and shrinkage changes in $[Ca^{2+}]_i$ and pH_i occur simultaneously, which raises the question whether these changes are interrelated or independent. $[Ca^{2+}]_i$ increased in isotonic media after addition of thapsigargin, but this increase in $[Ca^{2+}]_i$ was not accompanied by a change in simultaneously measured pH_i (Fig. 3A and Table 3). Incubation of PT cells in isotonic nominally free Ca^{2+} medium reduced $[Ca^{2+}]_i$ and concomitantly decreased pH_i (Table3). Incubation of PT cells in an isotonic medium at pH 8.5 raised pH_i and simultaneouslyincreased $[Ca^{2+}]_i$ (Fig. 5A and Table 3). On the contrary, lowering the extracellular pH to 6.5 decreased pH_i as well as $[Ca^{2+}]_i$ (Fig 5B and Table 3). Changes in pH_i brought about by the NH_4Cl pulse method influenced $[Ca^{2+}]_i$ similarly as shown for extracellular pH changes (results not shown).

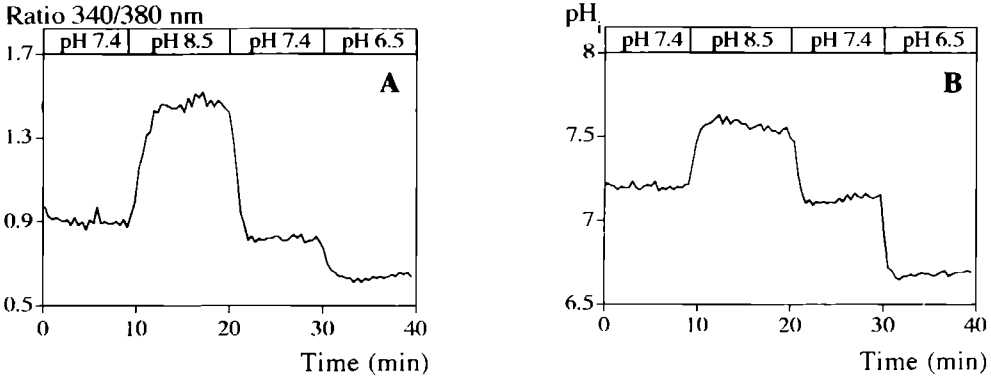


Figure 5

A. Typical response in fura-2 ratio in one cell in a confluent monolayer of rabbit PT cells after incubation in isotonic medium at pH 8.5, 7.4, and 6.5. The fura-2 ratio becomes 1.41 ± 0.05, 0.84 ± 0.02, and 0.67 ± 0.02 respectively (mean value ± SE; n=32).

B. Typical response in pH_i measured in the same cell as in Fig. 5A after incubation in isotonic medium at pH 8.5, 7.4, and 6.5. The pH_i becomes 7.82 ± 0.06, 7.34 ± 0.05, and 6.84 ± 0.04 respectively (mean value ± SE; n=32).

In addition, we used the Ca^{2+} chelator BAPTA-AM to buffer the Ca^{2+} peak generated by cell swelling. BAPTA already decreased resting $[Ca^{2+}]_i$ levels since the fura-2 ratio declined from 0.88 ± 0.02 to 0.63 ± 0.02. This decline in $[Ca^{2+}]_i$ was accompanied by a decrease in pH_i from 7.29 ± 0.05 to 7.11 ± 0.05. However, cell acidification still occurred after cell swelling despite the absence of the transient increase in $[Ca^{2+}]_i$ (Fig 6A, B). These results demonstrate clearly that during cell volume perturbations Ca^{2+} signals and pH_i can change independently.

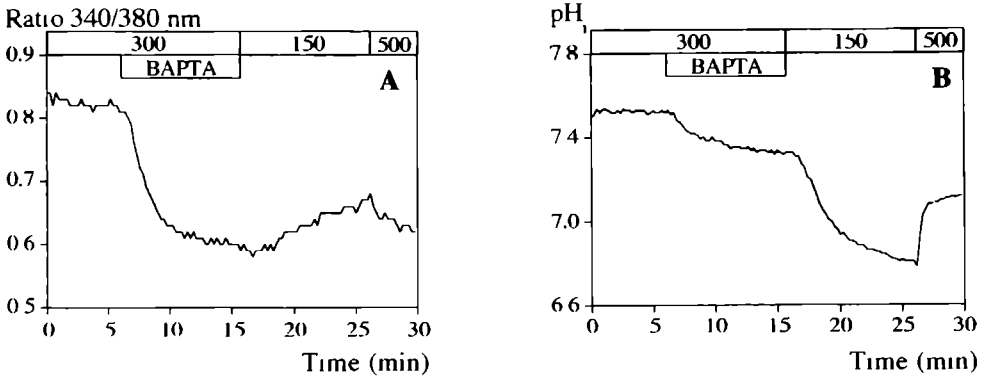


Figure 6

A Typical response in fura-2 ratio in one single rabbit PT cell in a confluent monolayer after loading for 10 min with 10 μM BAPTA-AM. No significant increase in fura-2 ratio was observed when the isotonic (300 mosM) medium was changed for a hypotonic (150 mosM) one

B Typical response in pH_i measured in the same cell as in Fig. 6A after loading for 10 min with 10 μM BAPTA-AM in isotonic (300 mosM) medium. The acidification after hypotonic (150 mosM) perturbation is still observed, despite the buffering of the Ca^{2+} spike

Table 3. Effect of pH_i perturbations on $[Ca^{2+}]_i$ and vice versa at isotonicity.

	FURA-2 RATIO		pH_i		
Condition	before	after	before	after	n
thapsigargin	0.87 ± 0.02	1.14 ± 0.04 *	7.32 ± 0.03	7.34 ± 0.03	32
Ca^{2+} free	0.89 ± 0.02	0.70 ± 0.03 *	7.39 ± 0.03	7.27 ± 0.03 *	48
pH_o 8.5	0.92 ± 0.02	1.41 ± 0.05 *	7.37 ± 0.04	7.82 ± 0.06 *	32
pH_o 6.5	0.84 ± 0.02	0.67 ± 0.02 *	7.34 ± 0.05	6.84 ± 0.04 *	32

Fura-2 ratio and pH_i were measured simultaneously under isotonic conditions. Initial values were compared with values directly after conditions in which 1 μM thapsigargin, nominally Ca^{2+} free medium (+ 0.1 mM $LaCl_3$) or KHB with high and low pH were present. Values are mean ± SE of n different cells from 2 or 3 separate isolations. * significantly different from value before experimental manipulation, $P < 0.05$.

DISCUSSION

In the present study we observed in PT cells in primary culture abrupt changes in $[Ca^{2+}]_i$ and pH_i upon osmotic perturbation. This raises the question whether changes in $[Ca^{2+}]_i$ and pH_i are involved in activating volume recovery mechanisms, or whether they result from transport processes activated by volume perturbation. After a hypotonic shock $[Ca^{2+}]_i$ increased transiently in PT cells which is in line with observations in other cell types [Pierce and Politis, 1990]. This rise in $[Ca^{2+}]_i$ has been postulated to start the process called RVD by activating K^+ and Cl^- channels [McCarty and O'Neil, 1992]. Also in isolated perfused rabbit proximal tubules $[Ca^{2+}]_i$ has been shown to play a role in RVD, although the $[Ca^{2+}]_i$ signal varies among different investigators [Suzuki *et al.*, 1990; Beck *et al.*, 1991; McCarty and O'Neil, 1991; Breton *et al.*, 1992]. In our study, no sustained Ca^{2+} level was observed as previously described by McCarty and O'Neil [1991] and Beck *et al.* [1991]. Differences may be due to a variation in experimental conditions. We used cultured PT cells and measured at 37° C, while Suzuki *et al.* [1990] also using cultured PT cells measured $[Ca^{2+}]_i$ at room temperature. In the other studies mentioned, isolated perfused tubules were used at 37 °C. In contrast, McCarty and O'Neil [1991] used proximal straight tubules (PST) instead of proximal convoluted tubules (PCT). Another variable has been the magnitude of the hypotonic shock which ranged from 50 to 150 mosM. In our study the observed Ca^{2+} spike was only partially dependent on extracellular Ca^{2+} which is in contrast to McCarty and O'Neil [1991] and Breton *et al.* [1992], who reported a complete dependence on extracellular Ca^{2+}. In the presence of thapsigargin we still observed an increase in $[Ca^{2+}]_i$ after hypotonic incubation, but the height of the peak was substantially reduced. Our results demonstrate that in cultured PT cells osmotic swelling mobilizes calcium from extracellular as well as intracellular sources.

In addition to the rapid increase in $[Ca^{2+}]_i$, cultured PT cells also slowly acidified upon cell swelling. Beck *et al.* [1992] previously reported an acidification in perfused proximal tubules, but in the presence of HCO_3^- a small alkalinization was observed. Intracellular acidification also occurred in osteosarcoma cells upon cell swelling independent of the presence or absence of HCO_3^- [Star *et al.*, 1992]. In addition, cell acidification after swelling was observed in human platelets [Livne *et al.*, 1987] and Ehrlich cells [Livne and Hoffmann, 1990]. After dilution of the intracellular fluid by swelling one would

expect an alkalinization, suggesting that in PT cells additional systems could be involved. In search for mechanisms that could explain the swelling induced cell acidification we could exclude Na^+/H^+ exchange and Cl^-/HCO_3^- exchange. Livne *et al.* [1987] suggested that increased metabolic acid production or K^+/H^+ exchange was responsible for the acidification. In Ehrlich cells Cl^-/HCO_3^- exchange plays a role in acidification after cell swelling [Livne and Hoffmann, 1990], while in osteosarcoma cells conductive efflux of OH^- was responsible [Muallem *et al*, 1992]. A conductive efflux could not be demonstrated in the present study, since depolarization did not affect acidification in cultured PT cells.

In contrast to osmotic swelling, osmotic shrinkage of cultured PT cells decreased $[Ca^{2+}]_i$. In nominally free Ca^{2+} medium, $[Ca^{2+}]_i$ decreased even further after a hypertonic shock suggesting that an abrupt stop in Ca^{2+} influx is not the mechanism behind this Ca^{2+} lowering event. Also increased uptake of Ca^{2+} by the ER can not explain the decline in $[Ca^{2+}]_i$, since thapsigargin did not prevent it. The only remaining possibility is an increased Ca^{2+} efflux, which is hard to prove experimentally in view of lack of specific inhibitors of plasma membrane Ca^{2+}-ATPases.

There are few studies reporting effects of hypertonicity on $[Ca^{2+}]_i$. In several studies, however, it was shown that RVI is independent of $[Ca^{2+}]_i$ [Grinstein *et al.*, 1982, Hoffmann *et al.*, 1983; Foskett and Spring, 1985]. In rat thymic lymphocytes, hypertonic activation of Na^+/H^+ exchange also caused a rise in $[Ca^{2+}]_i$ which was a secondary effect since $[Ca^{2+}]_i$ did not increase in sodium-free solutions. It is not very likely that a decrease in $[Ca^{2+}]_i$ serves as a signal for activation of volume recovery mechanisms since in general only Ca^{2+} spikes activate cells In gastric parietal cells Negulescu *et al.* [1992] observed a 30% decrease in $[Ca^{2+}]_i$ after cell shrinkage, but solely when $[Ca^{2+}]_i$ was first elevated by pre-treatment of carbachol Here, cell shrinkage was shown to be a selective inhibitor of Ca^{2+} influx. In our study, the decrease in $[Ca^{2+}]_i$ occurred without prior elevation of $[Ca^{2+}]_i$ by hormones

Cell shrinkage induced cytosolic alkalinization and the most likely explanation would be activation of Na^+/H^+ exchange, since this transport has been shown to be activated by hypertonicity in a variety of cell types [Hoffmann and Simonsen, 1989] Surprisingly, EIPA did not prevent shrinkage-induced alkalinization of cultured PT cells. However, EIPA did inhibit the recovery in pH_i after acidification by an NH_4Cl pulse. An explanation could be that an isoform of the Na^+/H^+ exchanger i.e. NHE-2 [Rosskopf *et al.*, 1993], which exhibits reduced

amiloride sensitivity and is present in the apical membrane of polarized cells is responsible for the shrinkage-induced alkalinization.

In cultured PT cells changes in $[Ca^{2+}]_i$ and pH_i take place simultaneously upon cell volume perturbation. The present study and other studies have shown that $[Ca^{2+}]_i$ and pH_i affect each other also in the absence of cell volume perturbations [Grinstein and Goetz, 1985; Siskind *et al.*, 1989; Daugirdas and Battle, 1992; Slotki *et al.*, 1993; Ziegelstein *et al.*, 1993]. The dependence of $[Ca^{2+}]_i$ on pH_i at isotonicity, appears to be tissue- and species-specific, since a change in pH_i have been shown to result in either a decreased, increased or unchanged $[Ca^{2+}]_i$ level [Grinstein and Goetz, 1985; Siskind *et al.*, 1989; Slotki *et al.*, 1993]. In vascular smooth muscle cells [Siskind *et al.*, 1989], rat lymphocytes [Grinstein and Goetz, 1985], chick cardiac myocytes [Kim and Smith, 1987] and in cultured PT cells in the present study, an increase in pH_i at isotonicity resulted in a rise in $[Ca^{2+}]_i$, while a decline in $[Ca^{2+}]_i$ was observed after lowering pH_i. On the contrary, in PT cells and in inner medullary collecting duct cells [Slotki *et al.*, 1993] a decrease in $[Ca^{2+}]_i$ at isotonicity resulted in an cytosolic acidification.

Despite this obligatory coupling of $[Ca^{2+}]_i$ and pH_i at isotonicity we could completely dissociate the rise in $[Ca^{2+}]_i$ induced by cell swelling from cytosolic acidification by using BAPTA. This Ca^{2+} chelator was able to prevent the rise in $[Ca^{2+}]_i$ upon osmotic swelling, but the acidification still occurred. In addition, at isotonicity an increase in pH_i was accompanied by an increase in $[Ca^{2+}]_i$ while upon cell shrinkage pH_i increased and $[Ca^{2+}]_i$ decreased. Therefore, changes in $[Ca^{2+}]_i$ and pH_i induced by cell volume perturbation are clearly dissociated. In addition, the time course of cell acidification is slower than the transient increase in $[Ca^{2+}]_i$. Also the fact that the increase in $[Ca^{2+}]_i$ was heterogeneous in contrast to the rather homogeneous decrease in pH_i is in support of uncoupled and independent phenomena.These observations suggest that $[Ca^{2+}]_i$ as well as pH_i play a role in the signal transduction cascade activated by cell volume sensors.

CHAPTER 3

Osmolarity-induced cellular calcium changes in vascular smooth muscle cells of spontaneously hypertensive rats

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Submitted for publication

SUMMARY

The hypothesis that altered sodium transport systems involved in cell volume regulation interact with cellular calcium homeostasis in vascular smooth muscle cells (VSMC) of Spontaneously Hypertensive Rats (SHR) was tested by measuring cytosolic free calcium concentration ($[Ca^{2+}]_i$) and $Na^+/K^+/2Cl$ cotransport activity during cell volume perturbation. $[Ca^{2+}]_i$ was measured in single VSMC of SHR and Wistar-Kyoto (WKY) normotensive rats in primary culture. An enhanced growth rate during cell culturing was observed in VSMC of SHR. Resting $[Ca^{2+}]_i$ was significantly ($P < 0.05$) higher in VSMC of SHR (136 ± 5 nM) when compared to WKY (116 ± 6 nM). On incubation in 150 mosM medium, $[Ca^{2+}]_i$ increased transiently to a significantly higher level in VSMC of SHR (285 ± 24 nM versus 209 ± 17 nM in WKY, $P < 0.05$). On incubation in 500 mosM medium, a rapid decrease in $[Ca^{2+}]_i$ was observed in VSMC from both rat types. After reaching a stable level, $[Ca^{2+}]_i$ was significantly higher in VSMC of SHR (116 ± 8 versus 74 ± 6 nM in WKY, $P < 0.05$). $Na^+/K^+/2Cl$ cotransport activity was determined by measuring bumetanide-sensitive ouabain-insensitive $^{86}Rb^+$ uptake. $Na^+/K^+/2Cl$ cotransport activity increased with increasing medium osmolarity (from 150 to 500 mosM) in both cell types. No significant differences in cotransport activity between VSMC of SHR and WKY were observed. Our data support the notion that cell volume regulatory mechanisms interact with Ca^{2+} homeostasis in VSMC and that this interaction is more pronounced in SHR than in WKY.

INTRODUCTION

The main function of arterial vascular smooth muscle cells (VSMC) is maintenance of tension by means of a dynamic equilibrium between contraction and relaxation. A disturbance of this delicate balance will lead to alterations in vascular tone and could play an important role in the pathogenesis of essential hypertension. Cultured VSMC, derived from Spontaneously Hypertensive Rats (SHR) and Wistar-Kyoto (WKY) normotensive rats, are often used as a model to study causes and mechanisms of essential hypertension [Bendhack *et al.*, 1992; Asano *et al.*, 1993]. Recently, two sodium-dependent transport systems ($Na^+/K^+/2Cl^-$ cotransport and Na^+/H^+ exchange) were found to be disturbed in VSMC of SHR [O'Donnell and Owen, 1988; Berk *et al.*, 1989]. Compared to WKY, $Na^+/K^+/2Cl^-$ cotransport was either found to be reduced or increased in VSMC of SHR [Kuriyama *et al.*, 1988; O'Donnell and Owen, 1988; Orlov *et al.*, 1992a]. Another important Na^+ transport mechanism, the Na^+/H^+ exchanger, was found to be enhanced in VSMC of SHR [Berk *et al.*, 1989; Scott-Burden *et al.*, 1989; Okada *et al.*, 1993]. Both sodium transport mechanisms ($Na^+/K^+/2Cl^-$ cotransport and Na^+/H^+ exchange) are known to play a role in cell volume regulation [Sarkadi and Parker, 1991]. Most cells are able to regulate their volume when exposed to anisotonic media. In a hypotonic medium, swollen cells reduce their volume towards control values by a process called 'regulatory volume decrease' (RVD). Conversely, after exposure to hypertonic medium, shrunken cells increase their volume by a mechanism known as 'regulatory volume increase' (RVI). In general, RVD and RVI are achieved by net extrusion or uptake of KCl, respectively followed by concomitant loss or uptake of cell water [Hoffmann and Simonsen, 1989; Sarkadi and Parker, 1991; McCarty and O'Neil, 1992].

After exposure to anisotonic media, changes in intracellular calcium concentration ($[Ca^{2+}]_i$) have been observed in a wide variety of cells [McCarty and O'Neil, 1992]. Furthermore, $[Ca^{2+}]_i$ appears to play a major role in activation of volume regulation after cell swelling [McCarty and O'Neil, 1992]. Elevation of $[Ca^{2+}]_i$ in VSMC may be responsible for a variety of pathological states including hypertension [Berridge, 1994]. Blaustein [1977] suggested that an elevation of intracellular sodium concentration ($[Na^+]_i$) in VSMC could cause an increase of $[Ca^{2+}]_i$ via the Na^+/Ca^{2+} exchanger. Increased $[Na^+]_i$ in platelets from type 2 (non-insulin-dependent) diabetic patients was associated with a diastolic

blood pressure ≥ 90 mm Hg [Tepel *et al.*, 1993]. Assuming a net Na^+ influx mediated by the $Na^+/K^+/2Cl^-$ cotransporter under physiological conditions, an increase in $Na^+/K^+/2Cl^-$ cotransport would lead to an increase in $[Na^+]_i$. An enhanced Na^+/H^+ exchange mechanism would also lead to an elevation of $[Na^+]_i$. The aim of this study was to investigate whether altered sodium transport systems involved in cell volume regulation interact with $[Ca^{2+}]_i$ homeostasis in VSMC of SHR. Therefore, $[Ca^{2+}]_i$ and $Na^+/K^+/2Cl^-$ cotransport were studied during cell volume perturbation in primary cultures of VSMC derived from SHR and WKY rats.

MATERIALS AND METHODS

Materials

$^{86}RbCl$ was purchased from New England Nuclear (N. Billerica, MA, USA). Bumetanide was from Leo Pharmaceutical Products (Ballerup, Denmark). N-2-hydroxyethylpiperazine N' 2-ethanesulfonic acid (HEPES) and sodium dodecyl sulfate (SDS) were from Research Organics (Cleveland, OH, USA). Gentamycin was purchased from Schering Corporation (Kenilworth, NJ, USA) and fetal calf serum (FCS) from Sera-Lab (Sussex, UK). Fura-2/AM and pluronic F127 were from Molecular Probes, (Eugene, OR, USA). All other chemicals were obtained from Merck (Darmstadt, Germany).

Solutions and media

An isotonic (~300 mosM) Krebs-Henseleit buffer (KHB) was used during experimental incubations, consisting of (in mM): 110 NaCl, 5 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 2 NaH_2PO_4!H_2O, 10 sodium acetate, 4 L lactate, 1 L-alanine, 10 D-glucose and 20 HEPES (calibrated with TRIS to pH 7.4). To obtain a hypotonic (~150 mosM) solution, the sodium chloride concentration in this medium was reduced to 35 mM. A hypertonic (~500 mosM) solution was made by adding 200 mM mannitol to the isotonic medium. The osmolarity of all media was measured with a freezing-point-depression osmometer (Osmette A, Precision Systems, Sudbury, MA, USA) and, when necessary, adjusted with mannitol. Dulbecco's modified Eagle's medium (Imperial, Hampshire, UK), supplemented with 10 μg/ml gentamycin (DME+), was used during cell isolation. Culture medium consisted of DME+, supplemented with 10 % (vol/vol) FCS, 1 % 100 x non-essential amino acids (Gibco, Paisley, UK) and 28 mM L-glutamine.

Isolation and primary culture of vascular smooth muscle cells

Primary cultures of VSMC were obtained from 6-10 weeks old (~200 g) SHR and WKY male rats. Rats were killed by cervical dislocation or CO_2 gassing. Aortic sections (from its ventricular origin to the branching of the renal arteries) were rapidly excised and placed in DME+ medium. The aortas were cleaned of fat and connective tissue, cut longitudinally and

segments of the medial smooth muscle layer were peeled away from the adventitial layer These segments were cut into fine pieces (1 x 1 mm) and placed in a 5 ml tube The pieces were allowed to pelletate by means of gravitation before the medium was replaced by DME+ (1 ml/aorta), supplemented with 1 mg/ml (405 U/mg) collagenase (Sigma, St Louis, MO, USA), 0 5 mg/ml elastase (Boehringer Mannheim, Mannheim, Germany) and 0 5 mg/ml soy bean trypsin inhibitor (Worthington Biochemical Corporation Freehold, NJ, USA) The tube was placed in a roll over rotor (~30 rpm) at 37 °C for approximately 1 hour VSMC were isolated by enzymatic dissociation during this period and then digestion was stopped by adding 10 % (vol/vol) FCS Cells were collected by centrifugation (5 min, 200 x g) After aspiration of the digestion medium the cells were resuspended in culture medium (2 5 ml/aorta) When used for ^{86}Rb uptake measurements, cells were seeded on 24-wells plates coated with fibronectin (2 6 aortas/plate) When used for $[Ca^{2+}]_i$ measurements, cells were seeded on plastic Aclar coverslips (Allied Signal, Pottsville PA, USA) coated with fibronectin (2 3 aortas/3 coverslips) Cells were cultured in a humidified chamber at 37 °C and 5% CO_2 Medium was changed after 3 days subsequently every 48 hours and the day before experiments were performed Cells were used 7 10 days after isolation, while still subconfluent

Measurement of $[Ca^{2+}]_i$

VSMC cultured on plastic Aclar coverslips (Ø 22 mm) were incubated for 1 h in DME+ medium containing 4 8 μM fura-2/AM 0 02 % (wt/vol) pluronic F127 3 6 % (vol/vol) FCS and 2 9 mM probenecid in a shaking waterbath at 37 °C The coverslip was loaded in a thermostatic Leiden" chamber [Rose *et al* , 1993], of which the volume was reduced to 200 μl by a perspex insert An inverted Diaphot microscope (Nikon, Tokyo, Japan) with a 40 x quartz oil immersion objective was used to capture fluorescence (emission wavelength 492 nm) on a low level charge-coupled device (CCD) camera The collected data was processed by TARDIS software on the MagiCal system (Applied Imaging, Tyne & Wear, UK), previously described in detail by Neylon *et al* [1990] The excitation wavelengths (340 or 380 nm) were selected using a rotating filter wheel (filter timing 5 seconds between two successive captures of 340/380 image pairs) and directed to the cell by a dichroic mirror (DM400) Video frames were averaged 16 times (hardware averaging) to reduce noise from the camera

Before video imaging started cells were superfused (1 5 ml/min) for 10 min with isotonic KHB medium at 37 °C During the first 5 min of video imaging the cells were superfused with isotonic KHB Then the medium was switched to hypotonic KHB for 10 min, followed by hypertonic KHB for 5 min before the medium was changed back again to isotonic KHB for another 5 min All media contained 0 3 mM probenecid to reduce dye leakage from the cells $[Ca^{2+}]_i$ was expressed as ratio of fluorescence ($R_{340/380}$), or calculated from this ratio by *in vivo* calibration *In vivo* calibration was performed by a 2-point calibration procedure using the equation derived by Grynkiewicz *et al* [1985] $[Ca^{2+}]_i = K_d \times \beta \times \{(R - R_{min})/(R_{max} - R)\}$, in which β is the ratio of 380 nm fluorescence at zero Ca^{2+} versus saturating Ca^{2+} concentrations and in which R is the ratio of fluorescence at 340 nm versus 380 nm R_{max} was determined by adding 2 μM ionomycin to the cells in the presence of 10 mM CaCl$_2$ To determine R_{min}, cells were perfused with Ca^{2+} free medium containing 2 mM ethyleneglycol-bis (β aminoethyl ether) N N N N tetraacetic acid (EGTA) and 2 μM ionomycin K_d represents the dissociation constant of Ca^{2+} and fura 2 which was assumed to be 224 nM [Grynkiewicz *et al* 1985] Ca^{2+}

insensitive fura 2 fluorescence and cellular autofluorescence were determined by adding 2 mM $MnCl_2$ to the cells in the presence of 2 μM ionomycin and were routinely subtracted from the fluorescence signals No significant differences in background fluorescence between VSMC of SHR and WKY were observed

Measurement of $^{86}Rb^+$ uptake

$Na^+/K^+/2Cl$ cotransport activity was measured as ouabain-insensitive and bumetanide-sensitive K^+ influx, using $^{86}Rb^+$ as a K^+ congener, according to previously described methods [O'Donnell and Owen, 1989, Orlov *et al*, 1992b, Raat *et al*, 1994] with slight modifications Cells grown on 24-wells plates were, after aspiration of culture medium, pre incubated for 5 min in 200 μl of 300 mosM KHB medium, containing 1 mM ouabain and in the presence or absence of 10 μM bumetanide The pre-incubation medium was subsequently replaced by the same amount of KHB (150, 300 or 500 mosM) to which, besides ouabain or bumetanide, 2-5 μCi/ml ^{86}RbCl was added After 5 min, $^{86}Rb^+$ influx was stopped by washing the plates 4 times with 400 μl ice-cold KHB medium, containing 1 mM ouabain and 10 μM bumetanide Plates were allowed to air dry before the cells were dissolved in 500 μl 0 05% (vol/vol) SDS The cell lysates were transferred to scintillation vials and subsequently radioactivity was assayed by liquid scintillation counting Using a immuno-globulin as a standard, the coomassie blue protein assay (Biorad, Munich, Germany) was used to determine a mean total protein concentration from 2 wells in each experiment $Na^+/K^+/2Cl$ cotransport activity was expressed as nmol K^+ per mg protein per min

Statistics

Data are presented as mean ± SE of n observations Statistical significance was evaluated with analysis of variance (ANOVA) All observations are from at least 3 experiments on cells derived from different isolations

RESULTS

Primary culture of VSMC

During the isolation and primary culture of VSMC, a few differences between SHR and WKY were observed. The medial smooth muscle layer thickness was, compared to WKY, increased in SHR, while the connection of the adventitial layer to the medial smooth muscle layer was tighter in WKY Compared to WKY, an enhanced growth rate of VSMC was observed in SHR. This difference in growth rate was reflected in the mean total protein concentration determined in the $^{86}Rb^+$ uptake experiments (SHR· 0 30 ± 0 03 (n = 9), WKY 0 14 ± 0.02 (n = 9) mg protein/ml; $P < 0\ 001$) The difference in growth rate, observed between SHR and WKY, is in agreement with previous studies [Berk *et al*, 1989, Scott-

Burden *et al.*, 1989], reporting a two to threefold increased rate of cell growth in cultures of VSMC from SHR.

$[Ca^{2+}]_i$ changes in VSMC of SHR and WKY

To investigate the possibility of an interaction between cell volume regulation and Ca^{2+} homeostasis, $[Ca^{2+}]_i$ in VSMC of SHR and WKY was measured using the fluorescent ratio probe fura-2 during RVD and RVI provoked by medium osmolarity changes. Compared to WKY, basal $[Ca^{2+}]_i$ was significantly higher in primary cultured VSMC of SHR (SHR: 136 ± 5 nM (n = 67), WKY: 116 ± 5 nM (n = 48); $P < 0.05$). When the osmolarity of the incubation medium was lowered to provoke RVD, an increase in $[Ca^{2+}]_i$ was observed. An increase in medium osmolarity to provoke RVI, resulted in a decrease in $[Ca^{2+}]_i$ (Fig. 1).

To study these responses in more detail, six ratio levels were defined (R1-R6, see Fig. 2) and used to quantify and compare the responses of VSMC from SHR and WKY (Fig. 3). Compared to WKY, the basal (R1) and peak (R2) $[Ca^{2+}]_i$ levels

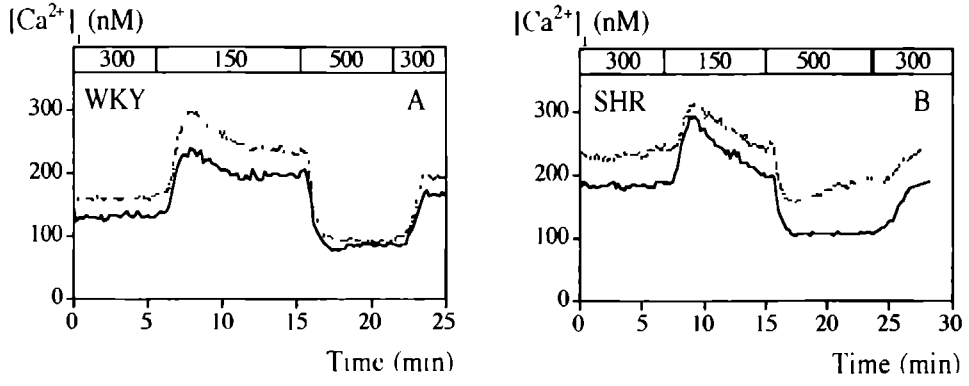

Figure 1

Effect of anisosmotic cell swelling and cell shrinkage provoked by medium osmolarity changes on $[Ca^{2+}]_i$ in WKY (**A**) and SHR (**B**) VSMC in primary culture. Two typical traces observed for both WKY and SHR are shown.

were significantly higher ($P < 0.05$) in SHR. The initial undershoot in $[Ca^{2+}]_i$ (R4), observed when the medium osmolarity was elevated, did not differ significantly ($P > 0.2$).

In most cells (~80%) from WKY, $[Ca^{2+}]_i$ decreased directly to a sustained level (R5) and the undershoot in $[Ca^{2+}]_i$ (R4) was observed in only a small number of cells. In VSMC from both SHR as well as WKY $[Ca^{2+}]_i$ decreased as medium

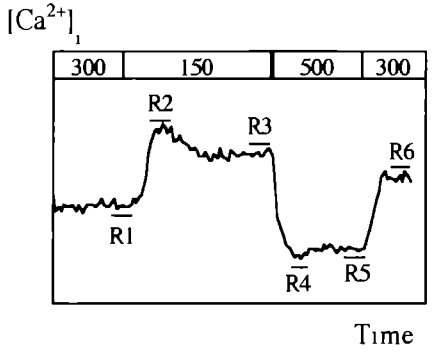

Figure 2
Effect of cell swelling and cell shrinkage on $[Ca^{2+}]_i$ in VSMC Definition of six $[Ca^{2+}]_i$ levels (R1-R6)

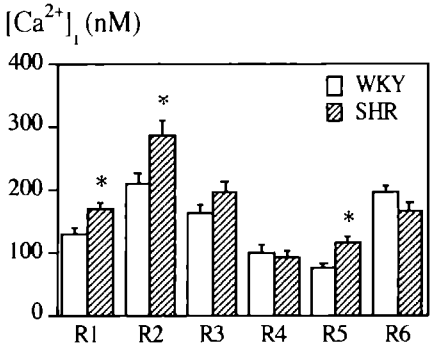

Figure 3
Effect of RVD and RVI provoked by medium osmolarity changes on $[Ca^{2+}]_i$ in WKY and SHR VSMC in primary culture R1-R6 are defined in Fig 2 Values are mean ± SE (n = 24 (WKY) and n = 23 (SHR), *P < 0 05)

osmolarity increased. At 150 mosM, the sustained $[Ca^{2+}]_i$ levels (R3) did not differ significantly (P > 0.2) between primary cultured VSMC from SHR and WKY. At higher osmolarities (300 mosM (R1) and 500 mosM (R5)), the $[Ca^{2+}]_i$ levels were significantly (P < 0.05) higher in VSMC from SHR

$Na^+/K^+/2Cl^-$ cotransport in VSMC of SHR and WKY

To compare $Na^+/K^+/2Cl$ cotransport between VSMC from WKY and SHR, K influx was studied by $^{86}Rb^+$ uptake measurements as described in 'materials and

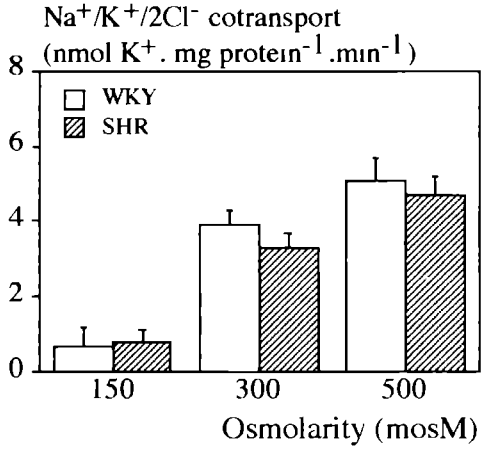

Figure 4
Dependence of $Na^+/K^+/2Cl$ cotransport activity on medium osmolarity in primary cultures of SHR and WKY VSMC Cotransport activity was measured as ouabain-insensitive and bumetanide sensitive K influx using $^{86}Rb^+$ as a K^+ congener Values are mean ± SE (n = 18) No significant differences between SHR and WKY were observed

methods'. The results of these experiments are presented in figure 4. $Na^+/K^+/2Cl^-$ cotransport activity, the rate of ouabain-insensitive and bumetanide-sensitive K^+ influx, increased with increasing medium osmolarity in both cell types. However, there were no significant differences in $Na^+/K^+/2Cl^-$ cotransport activity between SHR and WKY ($P > 0.2$).

DISCUSSION

To test whether cell volume regulation might interact with Ca^{2+} homeostasis, $[Ca^{2+}]_i$ was measured during cell volume perturbation. The initial increase in $[Ca^{2+}]_i$ observed during cell swelling, in VMSC of both SHR and WKY, is consistent with previous reports on other cell types [McCarty and O'Neil, 1992; Medrano and Gruenstein, 1993]. McCarty and O'Neil [1992], extensively discussed the role of calcium signalling during RVD in a recent review. More surprisingly, however, is the observed decrease in $[Ca^{2+}]_i$ during RVI in this study. This phenomenon has also been described for *Amphiuma* red blood cells [Cala *et al.*, 1986], and was observed in primary cultures of rabbit proximal tubule cells as well [Raat *et al.*, 1995]. So far, $[Ca^{2+}]_i$ has not been considered as a modulating mechanism in RVI [Hoffmann and Simonsen, 1989; McCarty and O'Neil, 1992].

In agreement with previous reports [Sugiyama *et al.*, 1990; Bendhack *et al.*, 1992; Asano *et al.*, 1993], basal $[Ca^{2+}]_i$ was significantly higher in aortic smooth muscle cells from SHR compared to WKY. In contrast to these findings, some investigators reported no differences in basal levels of $[Ca^{2+}]_i$ between these two strains, whereas, after hormonal stimulation a larger increase in $[Ca^{2+}]_i$ was observed in SHR [Nabika *et al.*, 1985; Neusser *et al.*, 1993]. In the present study, such an enhanced increase in $[Ca^{2+}]_i$ was also observed, but now upon incubation in hypotonic medium. In both VSMC cultures a decrease or increase in the sustained $[Ca^{2+}]_i$ levels, following the initial rapid increase or decrease, was observed when medium osmolarity was lowered or raised respectively. The difference in these $[Ca^{2+}]_i$ levels between both strains became more apparent at higher osmolarities, suggesting that sodium transport systems involved in RVI might interact with cellular calcium homeostasis.

One important sodium transport mechanism, involved in RVI in various cell types, is the $Na^+/K^+/2Cl^-$ cotransporter [Sarkadi and Parker, 1991]. In the present study, no significant difference in cotransport activity between primary cultured

VSMC of SHR and WKY was found. However, in contrast to these findings, a reduction in $Na^+/K^+/2Cl^-$ cotransport in VSMC from SHR was reported by O'Donnell and Owen [1988], using rats of equal weight and age. In both studies, cells were used while still subconfluent. In our study, however, primary cultures of VSMC were used whereas subcultures between the 5th and the 12th passages were used by O' Donnell and Owen [1988]. It is possible that during subculturing, slight differences between two cell types are amplified. On the other hand, this explanation is in opposition to observations reported by Berk *et al.* [1989]. They found that during serial passage, differences in growth rate and Na^+/H^+ exchange between SHR and WKY cells attenuated so that, by passage level 6, these differences were no longer present.

In human erythrocytes from patients suffering from essential hypertension, both increased as well as decreased $Na^+/K^+/2Cl$ cotransport activities have been reported [Garray *et al.*, 1980; Cusi *et al.*, 1981; Adragna *et al.*, 1982; Smith *et al.*, 1984]. Based on these observations, it was proposed that a defect in $Na^+/K^+/2Cl$ cotransport in VSMC could play a critical role in the etiology of essential hypertension. However, in contrast to these previous findings, no difference in cotransport activity between VSMC from SHR and WKY was found in our study. O'Donnell and Owen [1988] found a decrease in cotransport in VSMC of SHR, while two other studies showed an increase in $Na^+/K^+/2Cl^-$ cotransport in VSMC of SHR [Kuriyama *et al.*, 1988; Orlov *et al.*, 1992a]. Thus, whether a relationship exists between a defect in this transporter and the observed differences in $[Ca^{2+}]_i$ between SHR and WKY, remains inconclusive.

Besides $Na^+/K^+/2Cl$ cotransport, also Na^+/H^+ exchange could be involved in cell volume regulation after osmotic cell shrinkage in VSMC [Orlov *et al.*, 1992b]. Furthermore, the relative contribution of $Na^+/K^+/2Cl^-$ cotransport to RVI in VSMC is even less than that of the Na^+/H^+ exchanger [Orlov *et al.*, 1992b]. The disturbance of the Na^+/H^+ exchanger in SHR is very consistent in the literature. Several laboratories demonstrated an augmentation of Na^+/H^+ exchange in VSMC from SHR, compared to WKY [Berk *et al.*, 1989; Scott-Burden *et al.*, 1989; Okada *et al.*, 1993]. It is possible that the augmented Na^+/H^+ exchanger in SHR is responsible for the observed differences in $[Ca^{2+}]_i$ between SHR and WKY. Enhanced Na^+/H^+ exchange could lead to an elevation of $[Na^+]_i$ and intracellular pH (pH_i). An increased $[Na^+]_i$ as well as an increased pH_i can cause elevation of $[Ca^{2+}]_i$ [Blaustein, 1977; Siskind *et al.*, 1986]

Under physiological conditions, the osmolarity of the body fluids is kept

constant within narrow limits (~285 mosM ± 3%) [Hoffmann and Simonsen, 1989]. Thus, most cells are never confronted with threatening changes in the osmolarity of the extracellular milieu. However, apart from changes in extracellular fluid osmolarity, cell volume regulatory mechanisms may also play an important role in cell division and proliferation. Mitogens, like EGF and athrombin, stimulate $Na^+/K^+/2Cl^-$ cotransport in hamster fibroblasts [Paris and Pousségur, 1986]. In mouse 3T3 cells, amiloride (an inhibitor of Na^+/H^+ exchange) combined with bumetanide completely blocked the insulin stimulated transition from the G0/G1 phase to the S phase of the cell cycle [Panet *et al.*, 1986]. Furthermore, expression of the Ha-ras oncogene has been reported to stimulate $Na^+/K^+/2Cl^-$ cotransport and Na^+/H^+ exchange in NIH 3T3 fibroblasts, probably caused by a shift of the set point for volume regulation [Lang *et al.*, 1992]. Häussinger and Lang [1992], demonstrated that hormone-induced changes in cell volume can cause a shift between anabolic-proliferative and catabolic-anti-proliferative cellular metabolism. Thus, current evidence suggests that activation of $Na^+/K^+/2Cl^-$ cotransport and Na^+/H^+ exchange, both involved in RVI, are coupled to cell growth and maturation. Compared to WKY, we observed an increased medial smooth muscle layer thickness and an augmented cell growth rate in SHR, which corresponds well with previous studies [Berk *et al.*, 1989, Scott-Burden *et al.*, 1989]. These observations could imply that the increased growth rate, observed in SHR, is the cause of the observed differences in $[Ca^{2+}]_i$ homeostasis of VSMC from SHR and WKY upon medium osmolarity perturbation.

In conclusion, the alterations in Ca^{2+} handling, observed when cells are exposed to medium osmolarity perturbation, support the idea that sodium transport systems involved in RVI interact with calcium homeostasis. Since $Na^+/K^+/2Cl^-$ cotransport in VSMC was similar in SHR and WKY, another transport system, such as the Na^+/H^+ exchanger, is probably more involved in this process. On the whole, the results of the present study indicate that the observed increase in cell growth rate and the altered $[Ca^{2+}]_i$ homeostasis, in VSMC from SHR could be linked via altered cell volume regulatory mechanisms.

CHAPTER 4

Volume perturbations of proximal tubular cells in primary culture measured by cell thickness monitoring, trapped fluorescent probes, and Coulter counter

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SUMMARY

Osmotic cell volume perturbations of rabbit proximal tubule (PT) in primary culture were measured using three independent techniques. Automatic cell thickness monitoring of PT monolayers revealed that cell volume rapidly increased by 39 ± 2% in hypotonic medium (150 mosM) which was followed by partial regulatory volume decrease, RVD. Subsequent incubation in hypertonic medium (500 mosM) rapidly decreased cell volume by 54 ± 2% not followed by regulatory volume increase, RVI. When cell volume in PT monolayers was derived from concentration changes in the trapped fluorescent dyes, fura-2 or BCECF, osmotically induced cell volume changes appeared much smaller (17 ± 1% and 22 ± 2% for similar hypo- and hypertonicity, respectively). However, changes in fluorescence intensity were most often not in agreement with anticipated cell volume changes. Using the Coulter counter, a much larger shift in cell volume was observed in PT cell suspensions. In this situation, cell swelling in hypotonic medium amounted to 74 ± 2%, but was still followed by partial RVD. Hypertonicity resulted in a decrease in cell volume of 42 ± 3% not followed by RVI

In conclusion, our study indicates that automatic cell thickness monitoring of an epithelial cell layer cultured on a permeable support is a superior method and provides more reliable data than monitoring changes in fluorescence intensity of trapped dyes. The Coulter counter technique which uses a suspension of cells yielded the highest cell volume increase in PT cells, most likely due to lateral extension and unfolding of the basal labyrinth of isolated PT cells.

INTRODUCTION

Maintaining a constant cell volume is important for proper cell functioning and it is, therefore, not surprising that most mammalian cells are capable of regulating cell volume, not only in isotonic environments, but also as a reaction to osmotic perturbation. After cell swelling most cells restore their volume by a process called regulatory volume decrease (RVD) and in analogy hypertonically shrunken cells recover volume by regulatory volume increase (RVI) [Eveloff and Warnock, 1987; Hoffmann and Simonsen, 1989; Lewis and Donaldson, 1990].

In recent years it has become clear that cell volume regulation is more complex than originally anticipated. A wide variety of transport systems have been shown to be involved in cell volume regulation and most of them are probably regulated by volume-sensitive signal transduction pathways [Pierce and Politis, 1990; Sarkadi and Parker, 1991]. Despite the fact that most mammalian cells are normally not exposed to large differences in extracellular osmolarity, cell volume can also be challenged at isotonic conditions, for example, by hormonal activation of transporters or during cell division [Hoffmann and Simonsen, 1989]. Regulation of cell volume and of the transport mechanisms involved, varies widely among different cell types [Macknight, 1988]. To study the role of signal transduction pathways in cell volume regulation both parameters should, therefore, be measured at the single cell level. The techniques to measure concentration changes in intracellular ions at the single cell level have improved enormously in recent years. However, accurate measurement of cell volume changes remained difficult and depends largely on the cell type under study.

Until now, most techniques measure cell volume in a suspension of cells, like the Coulter counter which is most often used with blood cells [Livne *et al.*, 1987], but has also been used in cell suspensions obtained by trypsinization of attached cells [Roy and Sauvé, 1987; Ehrenfeld *et al.*, 1994]. Measuring cell volume in confluent monolayers on a substrate is technically more demanding. Previously, morphometrical methods as the change in tubule diameter have been used [Beck *et al.*, 1991], however, this method does not allow to measure changes in cell volume at the single cell level. Recently, an automatic cell thickness measuring system was developed by Van Driessche *et al.* [1993] in which the thickness of a single cell within an epithelial monolayer was monitored by alternatively focusing on the apical and basal membrane of the cell which were marked with fluorescent microbeads. Another method to monitor cell volume

changes has been described by Tauc *et al* [1990] and by Muallem *et al* [1992] and consists in measuring the intensity of a trapped fluorescent dye In theory, the last method provides an opportunity to measure simultaneously changes in $[Ca^{2+}]_i$ or pH_i and cell volume in a single cell

In the present study we have compared three methods i.e automatic cell thickness monitoring, measuring trapped dye concentrations, and the Coulter counter to evaluate their reliability and usefulness in studying cell volume regulation in rabbit PT cells in primary culture

MATERIALS AND METHODS

Materials

All chemicals were obtained from Merck (Darmstadt Germany) unless otherwise specified Bumetanide was obtained from Leo Pharmaceutical Products (Ballerup, Denmark) Fetal calf serum (FCS) was purchased from Sera Lab (Sussex UK) HEPES and TRIS from Research Organics (Cleveland, OH, USA) Gentamycin was obtained from Schering Corporation (Kenilworth, NJ, USA) Fura-2-AM, BCECF AM and fluorescent microbeads were from Molecular Probes (Eugene, OR, USA)

Solutions and media

The isotonic (300 mosM) medium was a Krebs Henseleit buffer (KHB) which contained (in mM) 110 NaCl, 5 KCl, 2 NaH_2PO_4, 1 2 $MgSO_4$ 10 sodium acetate 4 L lactate 10 D glucose 1 L alanine, 20 HEPES, and 1 $CaCl_2$ calibrated with TRIS to pH 7 4 Hypotonic (150 mosM) medium was similar to this solution except that NaCl concentration was reduced to 50 mM Hypertonic medium (500 mosM) was isotonic buffer to which 200 mM mannitol was added The osmolarity of the solutions was checked with an osmometer (Osmette A Precision Systems, Sudbury MA USA) and marginally adjusted to the desired value with mannitol K_1 medium consisted of a 1 1 mixture of Dulbecco s modified Eagle s medium (Imperial, Hampshire, UK) and Ham's F12 medium (Gibco Paisley UK) supplemented with gentamycin (10 μg/ml), $NaHCO_3$ (25 mM), glutamine (14 mM) 0 5% (vol/vol) 100 x non essential amino acids (Gibco Paisley UK) insulin (5 μg/ml) transferrin (5μg/ml) hydrocortisone (50 nM) prostaglandin E_1 (70 ng/ml), triiodothyronine (5 pM) and Na_2SeO_3 (50 nM) and having a pH of 7 4

Primary culture of rabbit kidney proximal tubule cells

Rabbit kidney proximal tubule (PT) cells were isolated and subsequently cultured as described previously [Rose *et al* , 1993] Briefly, PT cells were immunodissected from rabbit kidney with monoclonal antibodies 85C8 and 101E12 Subsequently cells were seeded either on coverslips on culture flasks or on transparent filters which were all coated with rat tail collagen (~40 μg/cm^2) Cells were seeded at a density of 2 10^5 cells/cm^2 In Coulter counter experiments cells

were detached from the culture flasks by 3 min trypsinization with 1x Trypsin/EDTA solution (Gibco, Paisley, UK) Before seeding cells on filters, 1 ml of a 1 2000 dilution of fluorescent polystyrene latex sulphate microbeads with a diameter of 1 μm (Molecular Probes, Eugene, OR, USA, ref no L 5081) were applied to transparent Anopore membrane tissue culture inserts (Nunc Intermed, Roskilde, Denmark) Beads were allowed to attach for ~ 20 min and then the solution was removed and the filter was coated with rat tail collagen Subsequently, cells were seeded on the membrane at a density of 4 x 10^5 cells/cm^2 Cells were cultured to confluency in K_1 medium equilibrated with 5% CO_2 - 95% air at 37 °C During the first 24 hours of culture, K_1 was supplemented with 5% (vol/vol) FCS Medium was changed every other day and the day before experiments were performed PT cells were used 6 days after seeding

Measuring cell volume by automatic cell thickness monitoring

The experimental set up used for cell thickness monitoring has been described in detail by van Driessche *et al* [1993] In brief, the filter with labelled cells was cut out of the culture insert and mounted in an Ussing-type chamber modified from van Driessche *et al* [1993] Compared to the original chamber, two closed compartments were used Fig 1 shows a schematic diagram of this modified Ussing type chamber The lower compartment was only 1 6 mm thick and the chamber was used upside down Both sides were closed with a cover slip Immersion oil was applied between the objective and the upper cover slip Since the working distance of the objective is 1 9 mm, the tissue could be easily focused Before experiments were started, a layer of biotin labelled carboxylate-modified latex microbeads (Molecular Probes, Eugene, OR, USA, ref no L 5251) was applied in a 1 500 dilution to the apical side of the cell monolayer and allowed to attach for about 15 min, resulting in a density of about 600 beads/mm^2 Subsequently, the Ussing chamber was mounted on the stage of the microscope and the tissue was superfused for 10 min at 37 °C at both sides with isotonic KHB at a rate of 3 ml per min to remove unattached microbeads After changing to a hypotonic buffer the position of the upper fluorescent beads together with a selected lower reference bead were recorded by a video camera coupled to a computer From the images the focal plane could be calculated by a fluorescent light intensity algorithm [Van Driessche *et al*, 1993] and the objective was automatically focused by changing its position by a piezo electric translator The displacement of the objective was used as a measure for the change in cell thickness By a modification in the original software up to 10 different beads can be selected in an image field This method has an accuracy higher than 0 1 μm [Van Driessche *et al*, 1993] and a time resolution between 5 and 8 s depending on the thickness of the cell layer and the number of beads selected For calculation of a mean graph of experimental data, the experiment with the smallest time period was chosen as a basis Using linear regression the time points for the other experiments were calculated

Monitoring cell volume with trapped fluorescent probes

PT cells grown on coverslips were loaded with fura-2 AM for 1 hr in KHB medium (300 mosM) containing 5 μM fura 2 AM, 0 02% (wt/vol) pluronic F127 and 3 mM probenecid in a shaking waterbath at 37 °C After loading, the coverslip was transferred to a thermostatic Leiden" chamber [Ince *et al*, 1983] The volume of the chamber was reduced to 200 μl by a

perspex insert For experiments in which $[Ca^{2+}]_i$ and pH_i were measured simultaneously, cells were subsequently loaded in ~ 200 μl KHB medium containing 1 μM BCECF AM until fluorescence intensity equalled that of fura-2 (usually within 5 min) The chamber was mounted on the stage of a Nikon Diaphot inverted microscope equipped with a 100x quartz oil immersion objective (NA=1 3) to monitor single PT cells Fluorescence was captured by a low-level charge coupled device (CCD) camera and further processed by TARDIS software on the MagiCal system (Applied Imaging, Tyne and Wear, UK) The MagiCal system has been described in detail by Neylon *et al* [1990] Cells were superfused with KHB at 37 °C at a rate of 1 5 ml/min Probenecid (0 3 mM) was present in the buffers to reduce dye leakage from the cells Before experiments, cells were pre-incubated for 10 min with isotonic KHB The concentration changes in total fura 2 fluorescence obtained by summation of the 340 and 380 nm signal of fura 2 [Alonso *et al*, 1989] and the pH insensitive signal of BCECF excited at 440 nm were used to measure cell volume changes During cell volume perturbation fluorescence intensity of both probes was measured in a restricted area within a single PT cell During experiments fluorescence intensity declined due to photo bleaching and dye leakage Correction for this decrease in intensity was performed as described by Muallem *et al* [1992]

Measurement of cell volume using the Coulter counter

A suspension of trypsinized PT cells was kept on ice in a stock of 4 5 * 10^6 cells/ml 200 μl of this stock was allowed to warm up to 37 °C before mixing with 10 ml of hypo- or hypertonic buffer at 37 °C After 1, 5, 9, 14 and 20 min of incubation cell volume was determined Cells measured in K_1 medium containing HCO_3 were kept on 37 °C in a 5% CO_2 95% air atmosphere Cell volume was measured in a Coulter counter model ZF (Coulter Electronics Harpenden, UK) Each sample was measured for 30 s and the number of cells counted ranged between 20,000 and 30,000 Output of the Coulter counter was directed to a Coulter channelyzer (Coulter Electronics, Dunstable, UK) in which a size distribution of 100 channels was sampled The output was connected to an A/D converter (Maclab World Precision Instruments, Sarasota, FL, USA) and the output was written to file Data was smoothed by a binomial smoothing procedure using the software program Igor (Wavemetrics, Lake Oswego OR, USA) and the channel with the maximum number of cells was used as a measure for the mean volume Besides 300 mosM, calibration was also performed in a 150 and 500 mosM medium by using polymer latex particles with a diameter of 17 9 μm and a volume of 3 pl (Coulter, Luton, England) This calibration indicated that during incubation in hypotonic medium cell volume will be overestimated by about 10% due to the lower Na^+ concentration and smaller conductance of the hypotonic medium This difference was corrected for by multiplying the outcome of the hypotonic measurement by the ratio of the isotonic and hypotonic values Assuming that a linear relation between channel number and cell volume does exist, changes in cell volume were expressed as changes in percentage of channel number

Statistics

Measurements were performed on cells derived from at least three different preparations Statistical significance was determined by one way analysis of variance (ANOVA) or a paired t-test Data is presented as the mean ± SE

RESULTS

Measuring cell volume by automatic cell thickness monitoring

Cell thickness of primary cultured rabbit proximal tubule cells (PT) in a confluent monolayer was measured by an automatic monitoring system developed by Van Driessche *et al.* [1993] as described in material and methods. Since the water permeability of the apical membrane of A6 cells, used by Van Driessche *et al.* [1993], is extremely low it was sufficient to change only the osmolarity at the basolateral side to induce cell swelling. In the present study, PT cells were used of which the apical and basolateral sides are very water permeable Changing the osmolarity only on one side resulted in a transcellular water flow without change in cell volume. Therefore, osmolarity of the solutions had to be changed simultaneously at both sides. Since the monolayer is viewed from the apical side with a water immersion objective we encountered the problemthat during changing the apical solution, the image of the fluorescence beads was distorted and the software could no longer keep track of the beads. The Ussing type perfusion chamber as used by Van Driessche *et al.* [1993] was, therefore, adapted as displayed in Fig. 1, allowing a fast replacement at both sides of the objective. In contrast to A6 cells, PT cells in culture have a low transepithelial resistance, reflecting the leaky paracellular pathway of the proximal tubule and this has the disadvantage that transepithelial resistance during osmotic cell volume perturbation does not provide cell membrane conductance changes.

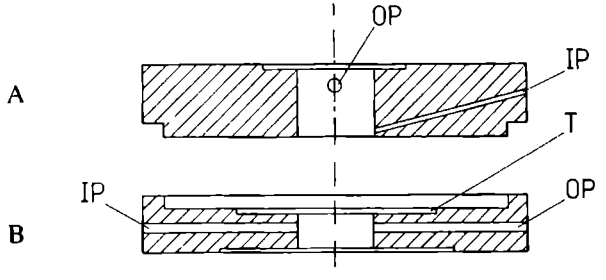

Figure.1

Scheme of the closed Ussing chamber used in automatic cell thickness monitoring, consisting of an upper (**A**) and bottom part (**B**) T, tissue PT cell grown on transparent filter, OP, outlet port for perfusion, IP, inlet port for perfusion

As shown in Fig. 2 and in Table 1, PT cell thickness rapidly increased when the monolayer was superfused with hypotonic KHB (150 mosM). In most cells this swelling was followed by a regulatory volume decrease which partly restored cell volume. Mean initial cell thickness was 10.5 ± 0.5 μm and cell

thickness increased by 39 ± 2% to 14.5 ± 0.7 μm. Due to RVD, cell thickness decreased with 23 ± 2% to 12.1 ± 0.6 μm after 10 min. When cells were subsequently superfused with hypertonic KHB (500 mosM) cell volume decreased rapidly by 54 ± 2% to a cell thickness of 5.9 ± 0.4 μm (Fig. 2, Table 1). No significant increase (6 ± 3%) in volume due to RVI was observed during 10 min incubation in hypertonic medium.

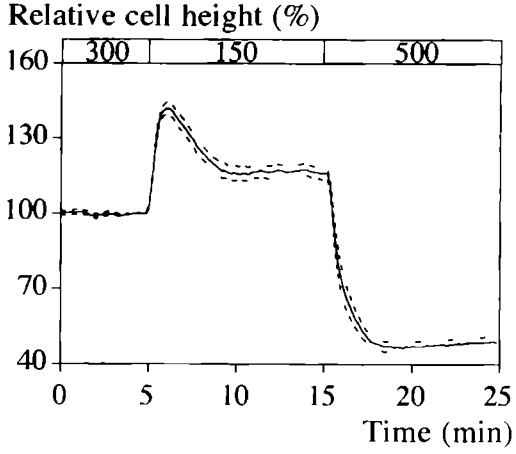

Figure. 2
Effect of hypotonic shock (150 mosM) and subsequent hypertonic (500 mosM) on cell volume in a confluent monolayer of rabbit proximal tubules in primary culture Relative changes in cell thickness were determined by automatic cell thickness monitoring Typical experiment showing the mean ± SE (dotted lines) of 5 experiments in which 10 individual cells were monitored in

Table 1 Relative change in cell volume after anisosmotic incubabtion of rabbit proximal tubule cells in primary culture

	VOLUME CHANGE (%)			
	hypotonicity	hypertonicity		
Osmolarity change (mosM)	300 -> 150	150 > 500	300 > 500	n
Method				
Automatic cell height monitoring	39 ± 2	54 ± 2	N D	70
Fluorescent trapped fura 2	17 ± 1	- 22 ± 2	12 ± 2	17
Fluorescent trapped BCECF	13 ± 1	- 15 ± 2	9 ± 2	22
Coulter coumter	74 ± 2	N D	42 ± 3	9

Initial changes in relative cell volume after 10 min incubation in hypotonic medium (150 mosM) and subsequent incubation in hypertonic (500 mosM) medium or after direct superfusion with 500 mosM medium With Coulter counting cell volume was measured directly and with the other two methods cell volume was based on changes in cell thickness or changes in ion insensitive fluorescence intensity of fura 2 or BCECF Change in fluorescence intensity was presumed to be linear with changes in cell volume N D – not determined

A remarkable heterogeneity was observed among different cells during swelling. On the same filter, one cell increased in volume by 18% while another exhibited a 88% increase. In addition, a heterogeneity in initial cell thickness was observed and the smallest cell thickness measured was 4 μm while the tallest cell amounted to 15 μm.

Monitoring cell volume by measuring trapped dye concentrations

Alterations in total, Ca^{2+}-independent, fluorescence of fura-2 and the pH-insensitive BCECF fluorescence excited at 440 nm were used to measure cell volume changes. To determine ion-insensitive fura-2 fluorescence the isobestic wavelength at 360 nm has been used in previous studies [Muallem *et al.*, 1992; Bibby and McCulloch, 1994]. We used both ratio probes simultaneously to measure, in addition to cell volume, changes in $[Ca^{2+}]_i$ and pH_i, but only 4 excitation wavelength filters could be placed in the filterwheel. Therefore, ion-insensitive fura-2 fluorescence was obtained from summation the 340 and 380 nm fluorescence signals, instead of the 360 nm signal. Figure. 3 indicates that the percentage change in total fluorescence intensity (340 + 380 nm) and in the isobestic fluorescence at 360 nm of fura-2 is equal.

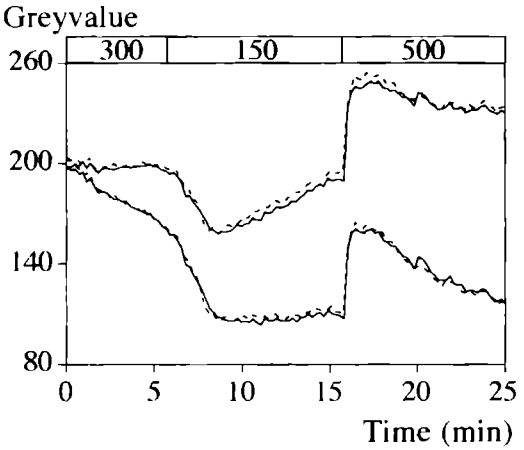

Figure 3

Corrected, Ca^{2+}-insensitive, fura-2 fluorescence as determined by adding the 340 and 380 nm signals (dotted line) and compared to the 360 nm signal. Uncorrected (bottom lines) and corrected (top lines) Ca^{2+} insensitive, fura-2 fluorescence (addition of 340 and 380 nm) after hypotonic (150 mosM) and subsequent hypertonic (500 mosM), incubation measured in a restricted area within a single PT cell. The rate constant for the decline in fluorescence was calculated by fitting the decline after the first 5 min in isotonic medium by a single exponential curve and this exponential was used to correct the signal for bleaching and dye leakage.

Also the shape of the two curves is similar and this is in agreement with Alonso *et al.* [1989].

During the experiment the intensity of the fluorescence declines due to photo bleaching and/or dye leakage This decline in fluorescence could be fitted to a single exponential curve [Muallem *et al*., 1992], but the time constant varied among individual cells and experiments. Therefore, for each individual cell its own decay rate constant was calculated from the first 5 min in isotonic medium and was used to correct the succeeding signal, as described previously by Muallem *et al.* [1992] An example of the changes in the uncorrected and corrected fura-2 fluorescence after osmotic perturbation is shown in Fig 3

Cell volume in an individual PT cell after osmotic perturbation was determined by measuring the changes in ion-insensitive fluorescence in a restricted area within the cell However, the changes in ion-insensitive fluorescence of fura-2 and BCECF were not always comparable, as would be expected In addition, cell volume changes derived from the 440 nm signal of BCECF are sometimes opposite of those derived from the fura-2 signal. This variation in results is shown in Fig. 4, which displays fluorescence signals from 2 different cells on the same coverslip containing fura-2 and BCECF Only 38% of the measured cells (n=45) showed a response which could unambiguously be interpreted as cell volume changes and these cells were further analyzed After hypotonic (150 mosM) superfusion these cells showed a rapid drop in total fura-2 fluorescence of 17 ± 1% (Table 1), which was followed by a partial recovery in the following 10 min incubation to 5 ± 2% below the initial fluorescence level Subsequent hypertonic (500 mosM) incubation increased fluorescence intensity with 22 ± 2% (Table 1) with a partial recovery in the following 10 min to 7 ± 1% above the initial level. Superfusion with hypertonic medium after isotonicity increased fluorescence by 9 ± 2% (Table 1) with no significant restoration of volume in the following 10 min.

Deriving volume changes from the pH-insensitive fluorescence of BCECF resulted in a decline in fluorescence of 13 ± 1% (Table 1) after hypotonic superfusion, with no recovery in the following 10 min Subsequent exposure to hypertonic medium increased fluorescence with 15 ± 2% (Table 1) with a partial recovery in the following 10 min to 7 ± 2% Superfusion with hypertonic medium preceded by isotonic conditions increased fluorescence intensity with 12 ± 2% (Table 1) followed by a partial restoration of cell volume to 5 ± 2% below the initial volume

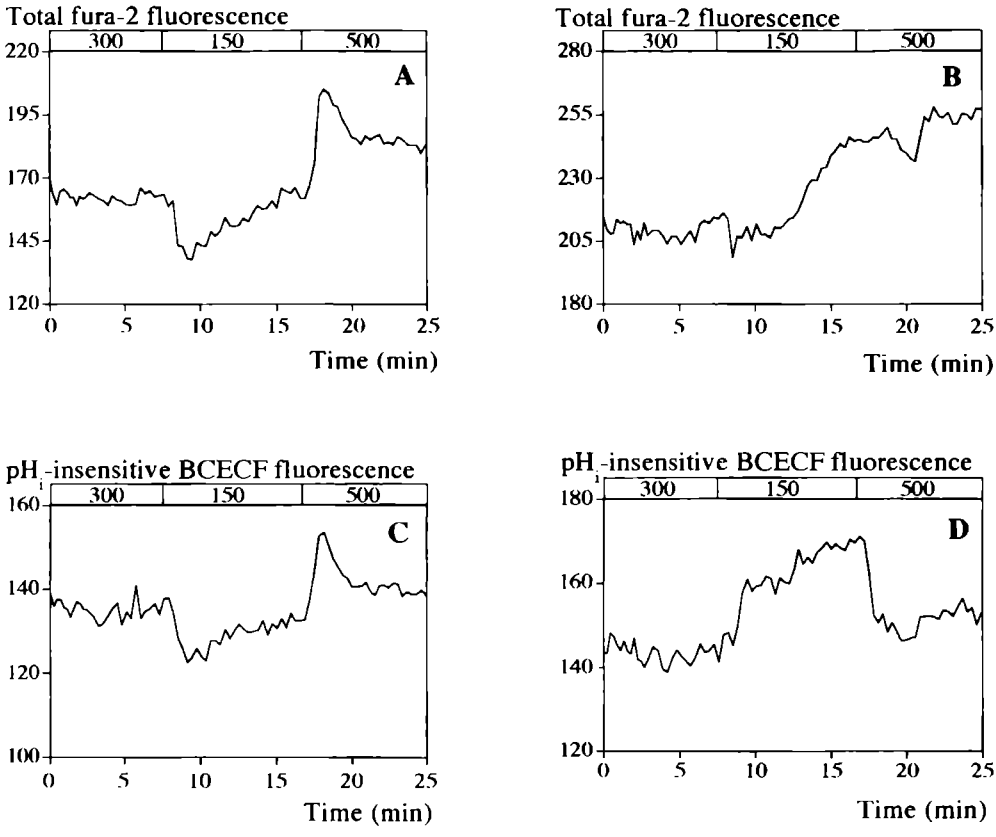

Figure. 4

Effect of medium osmolarity on the corrected signal of Ca^{2+}-insensitive fura-2 fluorescence (A, B) (summation of 340 and 380 nm signal) and simultaneously measured pH-insensitive BCECF fluorescence at 440 nm (C, D). Two different cells are depicted, one showing an anticipated (A, C) and the other a non-interpretable (B, D) cell volume response.

Monitoring cell volume in the Coulter counter

A suspension of PT cells (4.5 * 10^6 cells/ml) were either resuspended in hypotonic (150 mosM) or hypertonic (500 mosM) medium and incubation in isotonic medium (300 mosM) was used as a control. During incubation in isotonic medium no change in relative cell volume was measured over a period of 20 min (Fig. 5A,B). When cells were incubated in 150 mosM medium an initial increase in relative cell volume of 74 ± 2% (n=9) was measured which slowly decreased until it reached 33 ± 3% (n=8) after 20 min of incubation (Fig. 5A,

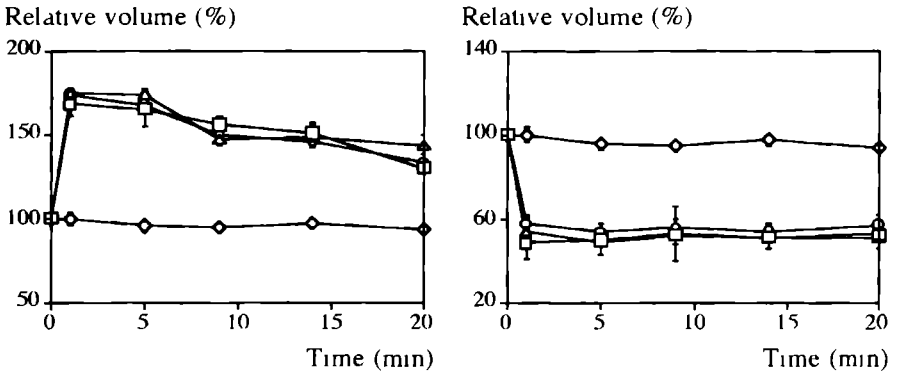

Figure 5

Effect of medium osmolarity on relative cell volume of cultured PT cells in suspension measured by the Coulter counter **A** PT cell volume increase after incubation in hypotonic 150 mosM medium (-O) Cells were also incubated in nominal Ca^{2+} free (+ 0 1 mM La^{3+}) 150 mosM medium (-Δ) and 150 mosM Ca^{2+} free medium containing 2 mM EGTA (□-) Response in isotonic 300 mosM medium (◇-) is shown as control Data is presented as the mean ± SE of at least 3 different preparations **B** PT cell volume decrease after incubation in hypertonic 500 mosM medium (O-) Cells were also incubated in 500 mosM K_1 medium with HCO_3 to which mannitol (Δ-) or NaCl (-□) was added to increase osmolarity Response in isotonic 300 mosM medium (-◇) is shown as control Data is presented as the mean ± SE of at least 3 different preparations

Table 1) In experiments with hypotonic nominally Ca^{2+} free medium to which 0 1 mM La^{3+} was added or in hypotonic Ca^{2+} free medium containing 0.2 mM EGTA, a RVD response was observed which was similar as in the presence of 1 mM Ca^{2+} (Fig. 5A). Incubation of cells in 500 mosM medium resulted in a cell shrinkage of 42 ± 3% (n=10) and no cell volume recovery was observed during a 20 min period (Fig 5B, Table 1). In 500 mosM K_1 medium, using either 200 mM mannitol or 100 mM NaCl, and containing HCO_3 , cells shrunk to the same extent (46 ± 8% and 52 ± 8%, respectively) as in KHB with HEPES. Also in HCO_3 containing solutions no RVI response was observed (Fig. 5B). Incubation of cells in 400 mosM medium resulted in a cell shrinkage of 28 ± 3% (n=4), but also with this smaller osmotic shock RVI did not occur (data not shown).

DISCUSSION

In the present study cell volume changes in single attached PT cells were determined using automatic cell thickness monitoring [Van Driessche *et al.*, 1993] and imaging of trapped intracellular fluorescent dye concentration [Tauc *et al.*, 1990; Muallem *et al.*, 1992; Bibby and McCulloch, 1994]. In addition, the Coulter counter was used to investigate whether differences exist between cell volume regulation in attached and suspension of PT cells.

With all three techniques, RVD was observed after swelling of PT cells in a 150 mosM medium, as has been reported in other studies [Roy and Sauvé, 1987; Suzuki *et al.*, 1990]. Compared to cell volume changes measured with automatic cell thickness monitoring, a much larger increase in cell volume was seen in the Coulter counter in media of equal osmotic strength. The difference between both methods may be explained by the fact that the lateral surface can expand of cells in suspension but not of cells in monolayers. This is especially valid in cell swelling, but absent in cell shrinking experiments. Indeed, exposure to a hypertonic medium of 500 mosM in the Coulter counter and in automatic cell thickness monitoring yielded similar cell volume changes.

Bibby and McCulloch [1994] measured cell volume in attached fibroblasts with fura-2 and in fibroblasts in suspension using the Coulter counter and found a similar discrepancy between attached cells and cells in suspension. Another difference between PT cells in a monolayer and in suspension is the time needed to restore cell volume. This phenomenon reflects the larger increase in cell volume in PT cells in suspension. Additional factors, however, delay RVD in suspended cells since RVD observed in the Coulter counter is more than twice as slow as RVD in attached PT cells. This may be indicative of damage of cytoskeletal elements in single isolated PT cells where lateral support is absent.

With the Coulter counter and with automatic cell thickness monitoring no RVI was observed in PT cells after cell shrinkage. The fluorescent signals, however, suggested a partial recovery after a hypertonic shock. This could be an artefact introduced by the correction for dye leakage since cell shrinkage might alter the time constant for dye leakage. To exclude that the osmotic shock differed too much from the physiological condition, we also performed a shock of 400 mosM, but again no RVI could be demonstrated with the Coulter counter technique. Also the literature is equivocal with respect to RVI in PT cells. Lohr and Grantham [1986] showed that PT cells maintain their volume when medium

osmolarity is gradually increased to 360 mosM, whereas a hypertonic shock of similar magnitude did not provoke RVI. In several cell types it has been shown that transport mechanisms involved in RVI have to be activated by a preceding RVD. This so-called pseudo-RVI or post-RVD-RVI has also been demonstrated in PT cells by Linshaw *et al.* [1992]. However, with two different techniques to measure cell volume we were unable to demonstrate RVI, even when PT cells were first exposed to a hypotonic shock and exhibited RVD. In a previous study, we have reported that cultured PT cells express $Na^+/K^+/2Cl^-$ cotransport which activity is dependent on medium osmolarity [Raat *et al.*, 1994]. From the present study we conclude, however, that $Na^+/K^+/2Cl^-$ cotransport is not contributing significantly to RVI in PT cells. It is, therefore, likely that $Na^+/K^+/2Cl^-$ cotransport capacity is too small to play a significant role in RVI.

Cell volume changes derived from changes in fluorescence intensity of trapped dyes were rather erratic in the present study. The method is based on the assumption that the total fluorescence in a single cell does not change when properly corrected for photo bleaching and dye leakage. By measuring the concentration of fluorescent dye in a restricted area within the cell an estimate of a change in cell volume can be made [Tauc *et al.*, 1990, Muallem *et al.*, 1992]. Tauc *et al.* [1990] were able to convert directly changes in fluorescence intensity into volume changes. In our study, cell volume changes measured with fluorescent probes were much smaller than in the study of Tauc *et al.* [1990]. A similar observation was made by Bibby and McCulloch [1994] using fura-2, who observed a volume change of 11% after a change from 300 to 150 mosM. Muallem *et al.* [1992] did not convert changes in fluorescent intensity into cell volume changes but used this method only in a qualitative way. When compared to automatic cell thickness monitoring, we conclude that changes in fluorescence intensity of trapped dyes underestimate cell volume changes in PT cells in monolayers.

There are several possibilities why cell volume measurements with fura-2 and BCECF fluorescence are less reliable. First, it is important to obtain a homogeneous dye loading. Compartmentalization of dye in intracellular organelles disturbs the reliability, since the permeabilities of intracellular membranes and the plasma membrane are likely to differ. Second, cell volume changes derived from two simultaneously present probes should yield similar results. However, in many experiments changes in BCECF and fura-2 intensities were different and even opposite which indicates that additional factors are of

influence on the fluorescence intensity of both probes. Third, accurate volume measurements requires a precise correction of the decay in fluorescence intensity due to photo bleaching and dye leakage. Despite the presence of probenecid during dye loading and experiments and in spite of restricted exposure to UV light, the fura-2 or BCECF fluorescence signals decreased in 25 min to 50% of the starting level. The fluorescence signal was corrected assuming an exponential decrease in fluorescence intensity in the first 5 min of the experiment at isotonicity. Volume perturbation, however, might influence the rate of dye leakage In support of this notion is the fact that fluorescence intensity decreased again after a hypertonic shock, while with the two other techniques no RVI was observed. In addition, in several experiments correction of the fluorescence signal resulted in an overestimation of the RVD or RVI responses

However, in certain conditions measuring cell volume with fluorescent probes could be an attractive technique that allows one to measure changes in cell volume simultaneously with changes in intracellular ions as Ca^{2+} and H^+. The present study indicates that this method underestimates cell volume changes Reliability may vary with the sort or size of the cell under study. Measuring fluorescence by confocal microscopy could well be a more reliable alternative since it precludes disturbance of the signal by fluorescence from other focal planes. Automatic cell thickness monitoring measures cell volume most straight forward, but this method is so far only applicable when confluent monolayers are under study.

CHAPTER 5

Regulation of $Na^+/K^+/2Cl$ cotransport activity in rabbit proximal tubule in primary culture

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SUMMARY

The presence of $Na^+/K^+/2Cl^-$ cotransport in rabbit kidney proximal tubule cells in primary culture was demonstrated by bumetanide-sensitive, ouabain-insensitive $^{86}Rb^+$ uptake studies. After addition of 10 μM bumetanide, $^{86}Rb^+$ uptake was inhibited from 11.1 ± 0.8 to 1.1 ± 0.1 nmol. mg protein^{-1} min^{-1} under isotonic (300 mosM) conditions. $Na^+/K^+/2Cl$ cotransport activities ranged from 2.2 ± 0.3 to 13.2 ± 1.0 nmol. mg protein^{-1} min^{-1} depending on the osmolarity of the medium (150 - 500 mosM). Decreasing extracellular pH to 6.5 inhibited, whereas increasing to 8.5 stimulated transport at all osmolarities. Decreasing intracellular pH (pH_i) by the NH_4Cl pulse method showed similar results, suggesting a possible regulatory role of pH_i on cotransport activity. Ca^{2+}-free medium increased cotransport activity 35 and 20% at iso- and hypertonicity, respectively. At 300 mosM, ionomycin (5 μM) inhibited cotransport by 25%. The combination of forskolin (10 μM) and 3-isobutyl-1-methylxanthine (1 mM) resulted in inhibition of cotransport activity by 38% at hypertonic conditions. Calyculin (1 μM) increased cotransport activity 134 and 128% at 150 and 300 mosM, respectively. In hypertonic medium calyculin did not influence cotransport activity. Okadaic acid (1 μM) had no effect on cotransport activity at all three osmolarities. NaF (10 mM) increased cotransport at all osmolarities tested.

INTRODUCTION

In absorbing and secreting epithelia large transcellular ion and water fluxes do exist For example, each minute a volume of water one to four times that of the cell volume is reabsorbed by proximal tubule (PT) cells [Lohr and Grantham, 1986] In PT cells Na /H' exchange is responsible for ~ 75% of total Na^+ influx. In general, the influx and efflux of ions are well balanced to prevent changes in intracellular volume When this balance is disturbed, for example, by a change in metabolic rate or by volume perturbation, volume regulatory mechanisms are necessary to restore cell volume Most cell types are capable of restoring cell volume after cell swelling or shrinkage by either regulatory volume decrease (RVD) or regulatory volume increase (RVI) mechanisms, respectively [Eveloff and Warnock, 1987; Hoffmann and Simonsen, 1989] In recent years, it has become clear that volume regulation is a dynamic process that is intertwined with several basic cellular functions including the regulation of a number of ion transport mechanisms [Hoffmann and Simonsen, 1989].

$Na^+/K^+/2Cl^-$ cotransport and Na^+/H^+ exchange have been shown to be involved in RVI in various cells [Eveloff and Warnock, 1987, Hoffmann and Simonsen, 1989] Although regulation of Na^+/H^+ exchange would be a very effective volume regulation system in PT cells, changes in the rate of this system could have further implications for intracellular pH (pH_i) regulation A preliminary study of Whittemburry *et al* [1992] has presented evidence for a $Na^+/K^+/2Cl^-$ cotransport system in the basolateral membrane of isolated perfused PT that plays a role in RVI It is possible that $Na^+/K^+/2Cl^-$ cotransport is recruited when other transporters cannot be activated or fail to restore cell volume Therefore, the presence and properties of $Na^+/K^+/2Cl^-$ cotransport were studied in PT cells in primary culture Since evidence is available that phosphorylation of the cotransporter plays a role in regulating its activity in various cells [Pewitt *et al*, 1990, Lytle and Forbush, 1992b], the putative regulation of $Na^+/K^+/2Cl^-$ cotransport activity in PT cells by protein-kinases, phosphatases, and second messengers was investigated during cell volume perturbation

MATERIALS AND METHODS

Materials

All chemicals were obtained from Merck (Darmstadt, Germany) unless otherwise specified $^{86}RbCl$ (20 mCi/ml) was purchased from New England Nuclear (N Billerica, MA, USA) Bumetanide was obtained from Leo Pharmaceutical Products (Ballerup, Denmark) Fetal calf serum (FCS) was purchased from Sera Lab (Sussex, UK), and N 2 hydroxyethyl-piperazine N'-2-ethanesulfonic acid (HEPES) and tris(hydroxy methyl)aminomethane (Tris) from Research Organics (Cleveland, OH, USA). Gentamycin was obtained from Schering (Kenilworth, NJ, USA), and ethyl-isopropylamiloride (EIPA) was from Molecular Probes (Eugene, OR, USA)

Solutions and media

The isotonic (300 mosM) incubation medium was a Krebs-Henseleit buffer (KHB) which contained (in mM) 110 NaCl, 5 KCl, 2 NaH_2PO_4, 1 2 $MgSO_4$, 10 sodium acetate, 4 L lactate, 10 D-glucose, 1 L-alanine, 20 HEPES, and 1 $CaCl_2$, calibrated with Tris to pH 7 4 Hypotonic buffers were similar to this solution except that Na^+ and Cl concentration were reduced to 50 mM resulting in a medium osmolarity of 150 mosM Hypertonic buffer (500 mosM) was isotonic buffer to which 200 mM mannitol was added Ca^{2+}-free buffer was medium without $CaCl_2$ to which 2 mM ethylene glycol-bis(b-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) was added NH_4Cl medium was KHB medium in which NaCl concentration was reduced to 80 mM and to which 30 mM NH_4Cl was added Na^+-free incubation medium was a modified 300 mosM KHB buffer which contained (in mM) 110 D(-) N-methylglucamine, 3 KCl, 2 KH_2PO_4, 1 2 $MgSO_4$, 10 acetic acid, 4 L lactate, 10 D-glucose, 1 L alanine 1 $CaCl_2$, and 20 HEPES, calibrated with Tris to pH 7 4 Cl -free incubation medium was a modified 300 mosM KHB buffer which contained (in mM) 110 $NaNO_3$, 5 KNO_3, 2 NaH_2PO_4, 1 2 $MgSO_4$, 10 sodium acetate, 4 L-lactate, 10 D-glucose, 1 L alanine and 1 $Ca(NO_3)_2$, and 20 HEPES, calibrated with Tris to pH 7 4 The osmolarity of the solutions was checked with an osmometer (Osmette A, Precision Systems, Sudbury, MA, USA) and adjusted to the desired value with mannitol Minimal K_1 medium consisted of a 1 1 mixture of Dulbecco's modified Eagle's medium (Imperial, Hampshire, UK) and Ham's F12 medium (Gibco, Paisley, UK), supplemented with gentamycin (10 μg/ml), $NaHCO_3$ (25 mM), glutamine (14 mM) and 0 5% (vol/vol) 100 x non-essential amino acids (Gibco, Paisley, UK), pH 7 4 K_1 medium was minimal K_1 medium supplemented with insulin (5 μg/ml), transferrin (5μg/ml), hydrocortisone (50 nM), prostaglandin E_1 (70 ng/ml), triiodothyronine (5 pM) and Na_2SeO_3 (50 nM)

Primary culture of rabbit kidney PT cells

Rabbit kidney PT cells were isolated and subsequently cultured as described previously in detail [Rose *et al*, 1993] Briefly, PT cells were immunodissected from rabbit kidney with monoclonal antibodies 85C8 and 101E12 Cells were seeded at a density of 2 x 10^5 cells/cm^2 on 24-wells plates that were coated with rat tail collagen (38 μg/cm^2) and cultured to confluency in K_1 medium During the first 24 h of culture, K_1 was supplemented with 5% (vol/vol) FCS Medium was changed every other day and the day before experiments were

performed PT cells were used 5-6 days after seeding Quiescent cells were obtained by incubation in minimal K_1 medium 20 h before experiments were performed

Measurement of $^{86}Rb^+$ uptake

The activity of $Na^+/K^+/2Cl^-$ cotransport in PT cells was determined by measuring the bumetanide sensitive $^{86}Rb^+$ uptake in the presence of ouabain to inhibit the Na^+/K^+ pump This method is based on the fact that Rb^+ replaces K^+ stochiometrically for transport by the cotransporter All incubations were performed at 37 °C Medium from the wells was aspirated, and cells were pre incubated for 5 min in 0 2 ml of 300 mosM KHB medium containing 1 mM ouabain and in the presence or absence of 10 μM bumetanide or other additions as specified in the results. In case of NH_4Cl pulse experiments, cells were incubated in NH_4Cl medium for 10 min followed by a 5 min incubation in 300 mosM KHB medium containing 10 μM EIPA Subsequently, the medium was replaced by 0 2 ml of the same KHB medium of the desired osmolarity, to which 0 5 μCi/ml $^{86}RbCl$ was added The $^{86}Rb^+$ influx was stopped after 3 min by washing four times with 0 4 ml ice-cold KHB medium to which 1 mM ouabain and 10 μM bumetanide were added Plates were allowed to air dry, and cells were subsequently lysed by adding 0 5 ml of 0 05% (vol/vol) sodium dodecyl sulphate to each well Radioactivity in the cell lysate was determined by liquid scintillation counting A mean total protein concentration, averaged from 4 wells, was used to express $^{86}Rb^+$ influxes per milligram protein Protein concentration was determined with the Coomassie blue protein assay (Bio rad Laboratories, Munich, Germany), using immunoglobulin G as a standard

Measurement of pH_i

PT cells cultured on 22 mm round coverslips were loaded with 2',7' bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) for 1 h in K_1 medium containing 2 μM of the acetoxymethyl ester of BCECF, 0 02% (wt/vol) pluronic F127, 4% (vol/vol) FCS, and 3 mM probenecid in a shaking water bath at 37 °C After the cells were loaded with BCECF, the coverslip with cells was transferred to a thermostatic Leiden chamber [Rose *et al*, 1993] The volume of the chamber was reduced to 200 μl by a Perspex insert The chamber was mounted on the stage of a Nikon inverted Diaphot microscope equipped with a 40x quartz oil immersion objective to monitor single PT cells Fluorescence was captured by a low-level charge coupled device camera and further processed by TARDIS software on the MagiCal system (Applied Imaging, Tyne and Wear, UK) [Rose *et al*, 1993] Cells were perfused with KHB buffer at 37 °C at a rate of 1 5 ml/min Probenecid (0 3 mM) was present in the buffers to reduce dye leakage from the cells Before experiments, cells were pre incubated for 10 min with isotonic KHB buffer pH_i was calculated from the 490/440 nm ratio of BCECF by calibration in a medium containing (in mM) 112 potassium gluconate, 28 KCl, 10 NaCl, 1 $MgCl_2$, 0 01 $CaCl_2$, 5 HEPES, 10 D-glucose, 20 mannitol and 0 01 nigericin From this buffer, a four-point calibration series between pH 6 0 and pH 7 8 was prepared by adjusting pH with Tris Calibration in eight single cells per coverslip from three different preparations were pooled, and a mean calibration curve was calculated Data points were fitted by linear regression to obtain a correlation between 490/440 nm ratio and pH_i

Regulation of $Na^+/K^+/2Cl$ cotransport activity

The involvement of intracellular calcium concentration ($[Ca^{2+}]_i$) in activation of cotransport was investigated by adding either ionomycin or EGTA. Compound R24571 (i.e., calmidazolium), an inhibitor of calmodulin-dependent processes, was used to study whether calmodulin-dependent protein kinase is involved. A possible role for protein kinase C (PKC) was studied by adding 12-O-tetradecanoylphorbol 13-acetate (TPA), a PKC activator or staurosporin which inhibits protein kinases. The role of protein kinase A (PKA) was investigated by raising the intracellular level of 3',5'-cyclic monophosphate (cAMP) by adding forskolin and 3-isobutyl-1-methyl xanthine (IBMX). 8-Bromoguanosine 3',5'-cyclic monophosphate (8-BrcGMP) was used to activate protein kinase G (PKG). Protein phosphatase activity was inhibited by incubating cells with okadaic acid or calyculin. The effect of activation of G proteins on cotransport activity was studied by adding either NaF or mastoparan [Higashijima *et al.*, 1988].

Statistics

All measurement were performed in duplicate on cells derived from at least three different isolations. Statistical significance was determined by one-way analysis of variance. Data are means ± SE.

RESULTS

Time course of bumetanide-sensitive $^{86}Rb^+$ uptake

The time dependence of $^{86}Rb^+$ uptake in PT cells was investigated in the presence of the Na^+/K^+ pump inhibitor ouabain (1 mM). Figure 1A shows that $^{86}Rb^+$ uptake was linear for up to 5 min both in the absence and presence of 10 μM bumetanide. At all time points, $^{86}Rb^+$ uptake was largely inhibited by 10 μM bumetanide. Therefore, in all subsequent experiments $^{86}Rb^+$ uptake was determined at 3 min, and this activity is presented as ouabain-insensitive bumetanide-sensitive $^{86}Rb^+$ uptake and is referred to as $Na^+/K^+/2Cl$ cotransport activity. In addition, dependence of $^{86}Rb^+$ uptake on extracellular Na^+ and Cl^- was determined. As shown in Fig. 1B, the absence of ionic Na^+ or Cl^- reduced $^{86}Rb^+$ uptake to similar levels as bumetanide. In Na^+-free medium $^{86}Rb^+$ uptake was not inhibited to the same extent as Cl^- free medium. The low Michaelis constant (K_m) of the cotransporter for Na^+ of ~ 0.6 mM [Wiener and Van Os, 1989] implies that residual levels of sodium can still influence the $^{86}Rb^+$ uptake. A dose-response curve is shown in Fig. 1C. The half maximal inhibition (IC_{50}) for bumetanide binding to the cotransporter was calculated to be 1.7×10^{-7} M, a value very similar to values found by others in different tissues [Haas, 1989, Wiener and Van Os, 1989].

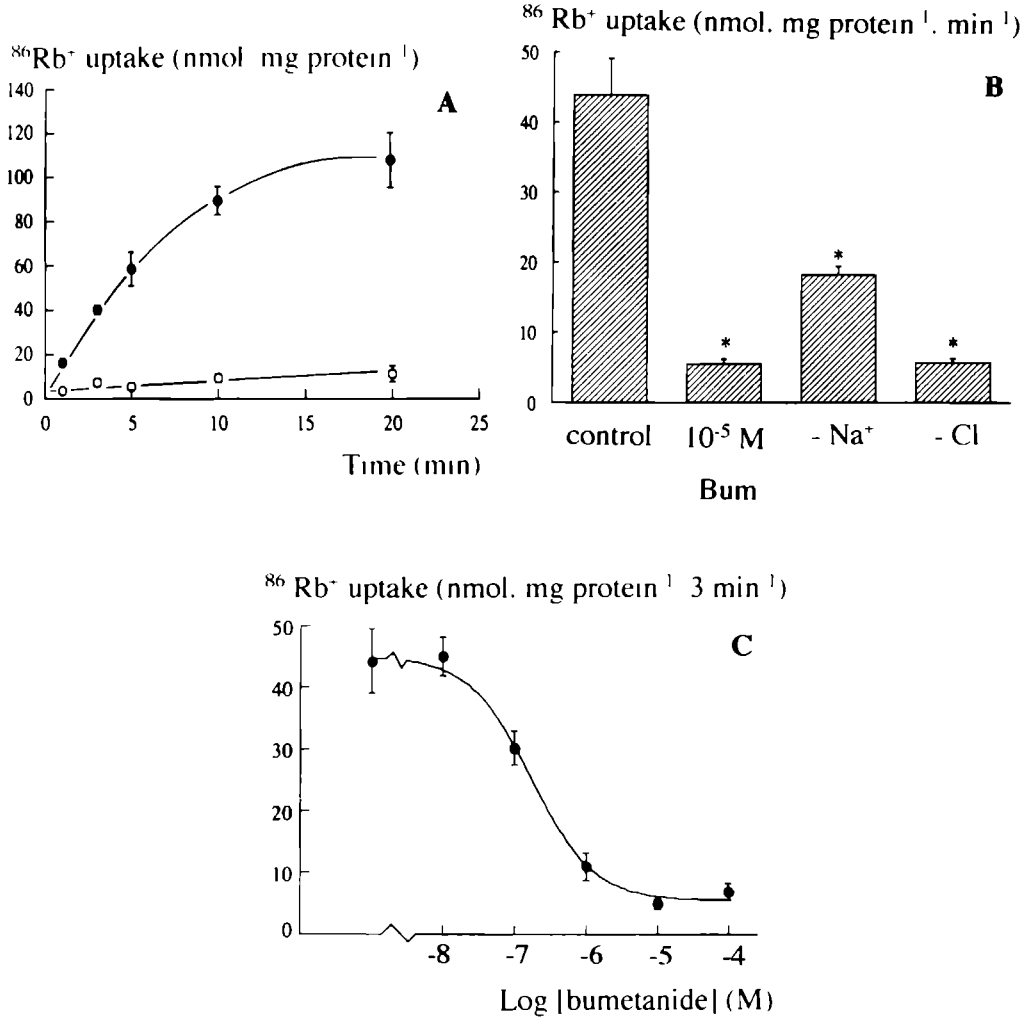

Figure 1

A.. Time-dependence of $^{86}Rb^+$ uptake determined in a primary culture of proximal tubule (PT) cells in the presence of 1 mM ouabain with (-O-) or without (-●-) 10 μM bumetanide. Values are mean ± SE of 3 separate experiments. **B.**: Dependence of ouabain-insensitive $^{86}Rb^+$ uptake on extracellular Na^+ and Cl in a primary culture of PT cells. For comparison the $^{86}Rb^+$ uptake in the presence of 10^{-5} M bumetanide is shown. Values are mean ± SE of 3 separate experiments. **C** : Dose dependent inhibition of $^{86}Rb^+$ uptake by bumetanide in a primary culture of PT cells , 10^{-5} M bumetanide was the most effective in inhibiting $^{86}Rb^+$ uptake. Curve in C was fitted by Michaelis-Menten kinetics yielding a half-maximal inhibition value of 1.7×10^{-7} M. For A-C, values are means ± SE of 3 separate experiments. *$P < 0.05$ compared with control.

During cell culture, several hormones such as insulin are present in the culture medium, which could influence $Na^+/K^+/2Cl$ cotransport activity. To test this possibility, PT cells were made quiescent by hormonal deprivation for 20 h before the experiment by incubation in minimal K_1 medium As can be seen in Fig. 2 this manoeuvre had a small, albeit significant, effect on $Na^+/K^+/2Cl$ cotransport activity at all three osmolarities. Since the presence of hormones reflects a more physiological situation in all experiments PT cells were cultured in K_1 medium.

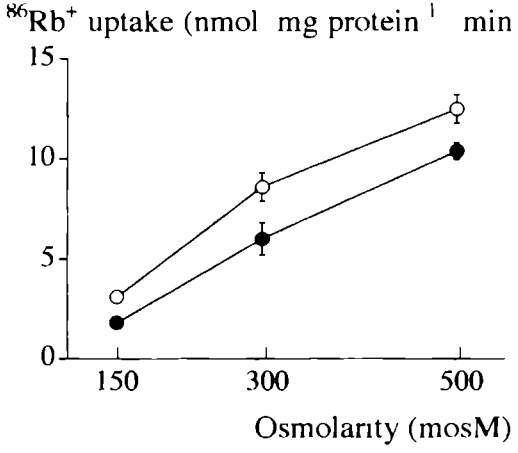

Figure 2
Dependence of ouabain insensitive, bumetanide sensitive $^{86}Rb^+$ uptake on medium osmolarity in a primary culture of PT cells $^{86}Rb^+$ influx was determined in cells exposed to minimal K_1 medium for 20 h before experiment (●-) or normal K_1 medium (-O) Values are mean ± SE of 3 separate experiments

Medium osmolarity and $Na^+/K^+/2Cl^-$ cotransport activity

The effect of medium osmolarity on $Na^+/K^+/2Cl$ cotransport activity was studied, and the results are shown in Fig. 2 Incubation of PT cells in a hypotonic medium of 150 mosM decreased $Na^+/K^+/2Cl$ cotransport activity, whereas incubation in hypertonic medium of 500 mosM resulted in a stimulation No significant effect of medium osmolarity on bumetanide-insensitive and ouabain-insensitive $^{86}Rb^+$ uptake was observed, except on incubation in a hypotonic medium of 150 mosM, which almost doubled bumetanide-insensitive $^{86}Rb^+$ uptake compared to isotonic conditions (data not shown).

Effect of pH on $Na^+/K^+/2Cl$ cotransport

The influence of acidification and alkalinization of the medium on $Na^+/K^+/2Cl$ cotransport was investigated, and the results are shown in Fig 3 At the three osmolarities tested incubation in low-pH (pH 6 5) medium inhibited cotransport, whereas a stimulation was seen at high pH (pH 8 5) At low pH $Na^+/K^+/2Cl$

cotransport activity decreased by 53, 62 and 90% under hypo-, iso- and hypertonic conditions, respectively. Conversely, in high-pH medium cotransport was stimulated by 95, 48 and 43%, respectively. Alteration of extracellular pH most likely results in changes in pH_i. Using BCECF, pH_i values of 6.7 ± 0.2 , 7.6 ± 0.2 and 8.1 ± 0.3 were measured in media with pH values 6.5, 7.4 and 8.5, respectively. To discriminate between an extra- or intracellular effect of pH on cotransporter activity, an NH_4Cl pulse procedure was performed [Roos and Boron, 1981], resulting in intracellular acidification from pH 7.5 ± 0.1 to 6.7 ± 0.1 at isotonic conditions, while extracellular pH was maintained at 7.4. Recovery of pH_i after the NH_4Cl pulse was prevented by the continuous presence of the Na^+-H^+ exchange inhibitor, EIPA. Under iso- as well as hypertonic conditions, NH_4Cl-induced intracellular acidification decreased cotransport activity, but no effect was observed at 150 mosM.

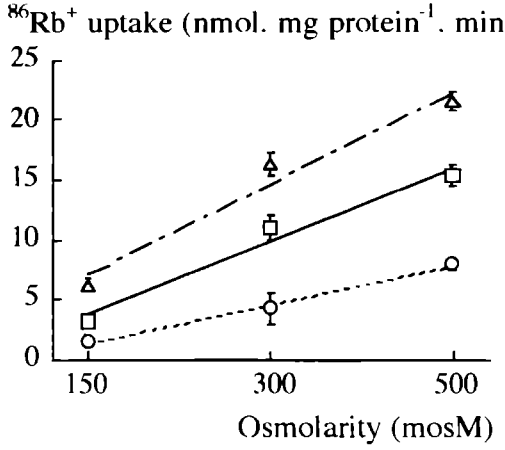

Figure 3
Dependence of ouabain-insensitive, bumetanide-sensitive $^{86}Rb^+$ uptake on extracellular pH in a primary culture of PT cells. Uptake was determined in 150, 300, and 500 mosM incubation medium at a pH of 6.5 (-O-), 7.4 (-□-) and 8.5 (-Δ-). Values are mean ± SE of 3 separate experiments.

Regulation of $Na^+/K^+/2Cl^-$ cotransport activity

To date, knowledge of the regulatory pathways involved in $Na^+/K^+/2Cl$ cotransport activation during cell shrinkage is scarce. Therefore, the effects of second messengers and activation of phosphorylation pathways on cotransport activity were investigated at hypo-, iso- and hypertonic conditions. In addition, the effect of direct activation of G proteins on $Na^+/K^+/2Cl$ cotransport was studied. Figure 4 shows the effect of possible $Na^+/K^+/2Cl$ cotransport modulators on cotransport activity. In isotonic medium, ionomycin (5 μM) inhibited $Na^+/K^+/2Cl$ cotransport activity, whereas no effect was observed in hypo- and hypertonic medium. Incubation in Ca^{2+}-free medium increased cotransport

activity at both iso- and hypertonic conditions, but not at hypotonic conditions. Incubation in hypertonic medium containing forskolin (10 μM) and IBMX (1 mM) reduced $Na^+/K^+/2Cl^-$ cotransport activity, whereas no effect was observed at iso- and hypotonicity. The protein phosphatase inhibitors okadaic acid (1 μM) and calyculin (1 μM) were tested for their effect on cotransport activity in PT cells. In iso- and hypotonic media, only calyculin stimulated cotransport activity. In hypertonic medium neither phosphatase inhibitors had an effect.

NaF (10 mM) stimulated $Na^+/K^+/2Cl^-$ cotransport activity at all three osmolarities. The effects of mastoparan (100 μM) were only determined at 300

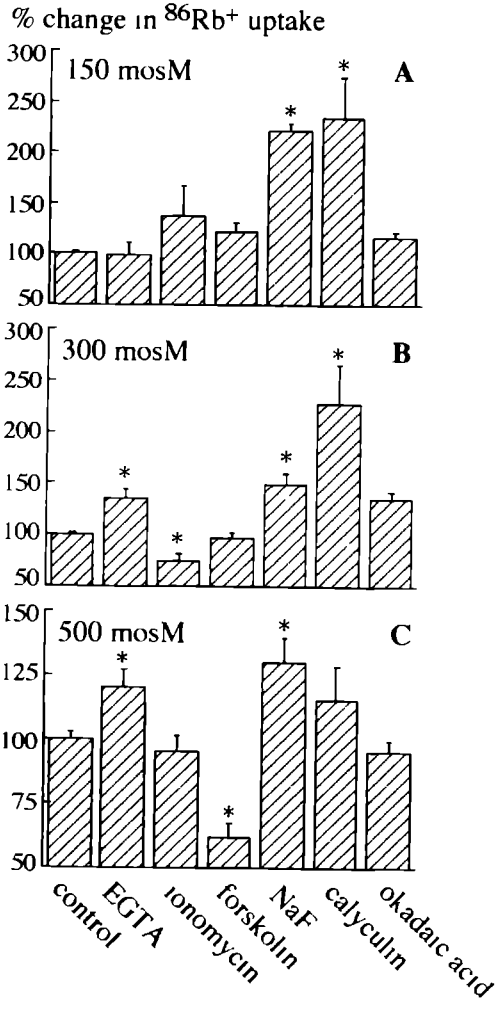

Figure 4

Effect of 0.1 mM EGTA, 5 μM ionomycin, 10 μM forskolin, 10 mM NaF, 1 μM calyculin and 1 μM okadaic acid on ouabain insensitive bumetanide sensitive $^{86}Rb^+$ influx in 150 (**A**), 300 (**B**), and 500 mosM (**C**) incubation medium in a primary culture of PT cells. Bars are means ± SE as percentage of control ± SE and were determined from 3 separate experiments. Control $^{86}Rb^+$ influxes were 2.2 ± 0.3, 10.0 ± 0.8 and 13.2 ± 0.9 nmol mg protein^{-1} min^{-1} in 150, 300 and 500 mosM medium respectively (*$P < 0.05$ compared to control).

mosM and in contrast did not influence cotransport activity. R24571 (10 μM), IPA (0.1 μM), staurosporin (0.5 μM) and 8-BrcGMP (0.1 mM) were all without effect on $Na^+/K^+/2Cl^-$ cotransport in the three osmotic conditions tested (data not shown).

DISCUSSION

In rabbit kidney PT cells in primary culture the presence of a $Na^+/K^+/2Cl^-$ cotransport mechanism was demonstrated for the first time by means of bumetanide-sensitive, ouabain-insensitive $^{86}Rb^+$ uptake studies. Furthermore, a strong dependence of $^{86}Rb^+$ uptake on the presence of either extracellular Na^+ or Cl^- was demonstrated, and an IC_{50} value of 1.7×10^{-7} M was calculated from a bumetanide dose-dependency curve. An inhibition of cotransport activity was found under hypotonic conditions, whereas a stimulation of cotransport was observed during hypertonicity. Orlov *et al.* [1992b] have also described this same dependency of cotransport activity on medium osmolarity in vascular smooth muscle cells. These findings correspond with a role of $Na^+/K^+/2Cl^-$ cotransport in volume regulation. Activation of cotransport in case of cell shrinkage would be a mechanism for NaCl and KCl influx which is followed by water influx and finally results in cell volume restoration. Cotransport was inhibited in hypotonic medium under which condition water influx via an active cotransporter would counteract cell shrinkage during RVD. In addition, cotransport was found to be dependent on pH_i. An increase in pH_i stimulated cotransport, whereas the opposite result was observed upon cell acidification. The effect of pH_i is consistent with earlier data reported for fibroblasts by Paris and Pouysségur [1986].

Although no information is available on how cell shrinkage leads to activation of $Na^+/K^+/2Cl^-$ cotransport, several levels in this process can be recognized [Lewis and Donaldson, 1990], of which two will be discussed. First, when cell volume is decreased by incubating cells in hypertonic media, there must be a sensor reacting to these volume changes. Second, via a cascade of reactions such a volume sensor would pass on a signal to the cotransporter, resulting in its activation. Signal transduction modulators and second messengers could act at the level of a volume sensor or directly at the level of the cotransporter by phosphorylation or inhibition of dephosphorylation. At present,

it is difficult to discriminate between the two levels of regulation since molecular details of a volume sensor and of the cotransporter are still largely unknown

Until now, a number of volume-sensing mechanisms have been proposed [Sarkadi and Parker, 1991; Watson, 1991] of which most include detection of cell swelling, but some could also be involved in detection of cell shrinkage. An interesting possibility is that cell volume perturbation leads to conformational changes in regulatory proteins connected to the cytoskeleton Additional regulatory proteins could also be present in the cytoplasm where their activity is concentration dependent (so-called macromolecular crowding [Parker, 1993]). During swelling of PT cells, it was demonstrated that the cytoskeleton plays a role in RVD [Linshaw *et al.*, 1992]. Although it is still unclear whether the cytoskeleton is also involved in detection of cell shrinkage, some evidence suggests that the cotransporter is linked to the cytoskeleton [Matthews *et al.*, 1992].

In passing the signal from a volume sensor to the $Na^+/K^+/2Cl$ cotransporter, a number of signal transduction pathways may be involved. An obvious mechanism for regulating cotransport activity would be the level of phosphorylation. Recently, it has been demonstrated for different cell types that this cotransporter can be activated by phosphorylation [Pewitt *et al.*, 1990; Lytle and Forbush, 1992b]. However, other mechanisms of activation are not excluded, since for Na^+/H^+ exchange it has been shown that activation during volume regulation was not related to an increase in phosphorylation [Grinstein *et al.*, 1992]. The possible involvement of the second messenger Ca^{2+} and a number of agents that could alter the phosphorylation state of the cotransporter have been investigated in the present study.

At isotonic conditions, the cotransport activity correlated inversely with $[Ca^{2+}]_i$, and opposite effects of ionomycin and Ca^{2+}-free medium were observed. Under hypotonic conditions, however, cotransport activity is already low, which may explain the absence of an additional inhibition of cotransport by $[Ca^{2+}]_i$. Surprisingly, under hypertonic conditions ionomycin did not inhibit the cotransporter, while an even more prominent effect should be anticipated when the cotransporter is maximally activated. At hypotonicity, Ca^{2+}-free medium did not stimulate cotransport which could mean that removal of extracellular Ca^{2+} during osmotic stress affected cell attachment and the cell volume sensing system. The fact that an increase in $[Ca^{2+}]_i$ under hypertonic conditions does not lead to a decrease in cotransport activity suggests a different Ca^{2+} sensitivity of

the mechanisms that regulate cotransport once activated by cell shrinkage.

Involvement of PKC, PKG, or calmodulin-dependent protein kinases in the activation of $Na^+/K^+/2Cl^-$ cotransport could not be demonstrated. An inhibitory effect of forskolin in combination with IBMX was observed at hypertonicity, whereas no effect was seen at iso- and hypotonic conditions. This result again suggests, as with $[Ca^{2+}]_i$, that cotransport activity is regulated differently after activation by cell shrinkage. At isotonicity, an increase in cAMP has been reported to have both stimulatory and inhibitory effects on cotransport depending on the cell type studied [Haas, 1989] In rat vascular smooth muscle cells [O'Donnell and Owen, 1986], human fibroblasts [Owen and Prastein, 1985] and erythrocytes [Garay, 1982], cotransport was inhibited after an increase in cAMP In duck erythrocytes, cAMP raises the set point of cell volume regulation [Geck and Heinz, 1985], but no direct role of cAMP on cotransport activation was assumed in these cells. A similar effect of cAMP may be present in PT cells since cotransport activity under hypertonic conditions in combination with increased PKA activity is comparable to the activity at isotonicity.

Decreasing protein dephosphorylation by either okadaic acid or calyculin resulted only for the latter in activation of cotransport under isotonic conditions. Okadaic acid [Bialojan and Takai, 1988] and calyculin both inhibit phosphatase I and IIa, but calyculin is a more potent inhibitor of phosphatase I [Ishihara *et al.*, 1989]. Activation of cotransport by calyculin and not okadaic acid suggests the involvement of type I phosphatase in cotransport activation. Recently, similar results were reported for cotransport activity in other cell types [Paulais and Turner, 1992; Klein *et al.*, 1993]. The finding that $Na^+/K^+/2Cl^-$ cotransport is a substrate of phosphatase I corresponds with the fact that phosphatase I is usually membrane bound, while phosphatase IIa activity is mainly cytosolic [Cohen and Cohen, 1989] Grinstein *et al* [1992] suggested that there is a balance between protein kinase and phosphatase activity at isotonicity resulting in a steady phosphorylation of the cotransporter They also reported consistent evidence that activation and deactivation patterns are complementary for transport mechanisms involved either in RVD or RVI. For cotransport, and also for Na^+/H^+ exchange, phosphorylation by a volume sensitive kinase system has been found to be the mechanism for activation [Grinstein *et al* , 1992; Klein *et al.*, 1993].

In the present study, a stimulation of cotransport activity was observed with NaF, suggesting that G proteins are involved in stimulation of cotransport. However, the G protein stimulator mastoparan, a peptide toxin of wasp venom

[Higashijima *et al.*, 1988], had no effect under isotonic conditions. An explanation could be the fact that mastoparan activates G_i proteins to a higher extent than G_s proteins [Higashijima *et al.*, 1988], suggesting that it is G_s proteins that are involved in cotransport stimulation. Since PKC activation had no effect on cotransport activity, it is unlikely that a G protein coupled to phospholipase C is responsible for the NaF effect. In general, caution is needed in interpreting effects of NaF, since at concentrations in the millimolar range fluoride is known to release Ca^{2+} from intracellular stores [Chabre, 1990]. Moreover, the active complex AlF_4^-, which competes with phosphate for binding to G proteins, also inhibits protein phosphatases, phosphorylases, and many adenosinetriphosphatases [Chabre, 1990]. However, comparison of the effects of calyculin with those of NaF precludes that NaF solely inhibits phosphatases in PT cells. Pewitt *et al.* [1990] demonstrated in avian erythrocytes that NaF does not result in the same extent of phosphorylation as seen with okadaic acid and they suggest a possible role for a G protein. Normally G proteins are an intermediate between hormone receptor activation and modulation of an effector, but there are indications that G proteins are not solely influenced by extracellular signals. GAP-43, a protein found in the neural growth cone and regulated by several cellular messengers, has been shown to stimulate guanine nucleotide exchange on a G protein [Strittmatter *et al.*, 1990]. It is feasible, therefore, that cell shrinkage could alter the conformation of proteins connected to the cytoskeleton, resulting in G protein activation. Recently, Lytle and Forbush [1992a] observed that several smaller proteins copurify with the 195-kDa cotransporter isolated from shark rectal gland. They speculated that some of these proteins could be part of the cytoskeleton associated with the cotransporter in the intact cell and modulate its function. Our data suggests that a G-protein dependent pathway, probably involving a G_s protein which does not lead to PKC activation, might be involved in activation of the cotransporter. A similar mechanism has recently been proposed for the Na^+/H^+ exchanger, another transporter involved in RVI. Davis *et al.* [1992] described a role for G proteins in the transduction of the cell shrinkage signal to activation of Na^+/H^+ exchange and showed that PKA and PKC were not involved. At present, a more specific method to modulate G proteins will be necessary to study the possibility of a direct effect of G proteins on $Na^+/K^+/2Cl^-$ cotransport activation.

Until recently, there was no evidence for the presence of $Na^+/K^+/2Cl^-$ cotransport in PT. A preliminary study from Whittemburry *et al.* [1992], however,

has demonstrated the presence of $Na^+/K^+/2Cl^-$ cotransport in the basolateral membrane of isolated perfused PT and suggested a role in RVI. This indicates already that $Na^+/K^+/2Cl^-$ cotransport is present in PT, but has to be activated by pre incubation in hyperosmolar solutions. In addition, when PT cells are cultured, as in thepresent study, cotransport activity could be even more expressed than in noncultured PT. Therefore, further studies are needed to identify $Na^+/K^+/2Cl^-$ cotransport in noncultured PT and to demonstrate that this cotransporter is involved in RVI.

In conclusion, $Na^+/K^+/2Cl^-$ cotransport activity in rabbit PT cells in primary culture is strongly dependent on medium osmolarity and pH. The regulation of the cotransporter involves several signal transduction pathways, including phosphorylation by PKA and activation of G proteins.

CHAPTER 6

Culturing induced expression of basolateral $Na^+/K^+/2Cl^-$ cotransporter BSC2 in proximal tubule, aortic endothelium, and vascular smooth muscle

N.J.H. Raat, E.Delpire, C.H.van Os, and R.J.M. Bindels

SUMMARY

So far, two isoforms of the neutral $Na^+/K^+/2Cl^-$ cotransporter have been cloned in mammals. One isoform, BSC1, mediates apical ion entry in the renal thick ascending limb of Henle and a second, BSC2, appears to be an ubiquitously expressed $Na^+/K^+/2Cl^-$ cotransporter [Delpire *et al.*, 1994]. In primary cultures of rabbit proximal tubule (PT), porcine aortic endothelial cells (PAEC), and rat vascular smooth muscle cells (VSMC) expression of the second isoform BSC2 was demonstrated by Northern blot analysis and bumetanide-sensitive $^{86}Rb^+$ uptake studies. A surprising finding was the complete absence of BSC2 in fully differentiated freshly isolated PT, PAEC and VSMC. So far, several studies reported modulation of $Na^+/K^+/2Cl^-$ cotransport activity by vasoactive substances and suggested a role for disturbed cotransport in the pathogenesis of essential hypertension, but all these observations were made in cultured cells. Our study indicates that caution is needed when conclusions on regulation of $Na^+/K^+/2Cl^-$ cotransport obtained in cultured cells are extrapolated to the tissue of origin.

INTRODUCTION

In recent years $Na^+/K^+/2Cl^-$ cotransport has been identified in a wide variety of cells [Haas, 1994], where it is either involved in transepithelial NaCl transport [Eveloff and Calamia, 1986] or in regulation of cell volume [Eveloff and Warnock, 1987; Hoffmann and Simonsen, 1989] A defect in $Na^+/K^+/2Cl^-$ cotransport activity was reported in erythrocytes of patients with essential hypertension [Garay *et al.*, 1980]. Subsequent studies pointed to differences in vascular smooth muscle cells (VSMC) cotransport activity in spontaneously hypertensive rats (SHR) [O'Donnell and Owen, 1988; Orlov *et al* , 1992a] when compared with the normotensive Wistar Kyoto (WKY) rat. Cotransport activity in VSMC was found to be under control of several vasoactive substances including angiotensin II [O'Donnell and Owen, 1986; Owen and Ridge, 1989], endothelin [Rosati *et al.*, 1990] and ANP [O'Donnell and Owen, 1986; Owen *et al* , 1990] In endothelial cells cotransport was found to contribute to more than half of the total K^+ influx which could be inhibited by ANP and other vasoactive agents suggesting a role for cotransport in mediating the effects of these agents on the vasculature [O'Donnell, 1989].

Measuring cotransport activity by $^{86}Rb^+$ uptake studies in intact blood vessels is technically difficult due to a delay in diffusion and to the presence of several cell types [Orlov *et al.*, 1992a]. Therefore, all experiments in which cotransport activity has been determined in VSMC and endothelial cells were done with cells in culture rather than in the native tissue An important question is whether cotransport activity in cultured cells reflects cotransport activity in vascular smooth muscle and endothelium in intact blood vessels. Therefore, we studied the expression of $Na^+/K^+/2Cl^-$ cotransport in cultured and in freshly isolated rat VSMC, porcine aortic endothelial cells (PAEC), and rabbit proximal tubule (PT). The increased knowledge of $Na^+/K^+/2Cl^-$ cotransport has culminated in the recent cloning of several isoforms of this cotransporter [Gamba *et al.*, 1994; Haas, 1994; Xu *et al.*, 1994]. In the present study we have used a cDNA probe derived from a putative basolateral $Na^+/K^+/2Cl^-$ cotransporter BSC2, which is likely to be an ubiquitously expressed isoform and to play a role in cell volume regulaton in non-polarized cells [Delpire *et al* , 1994]. The expression of BSC2 proved to be prominent in cultured VSMC, PAEC, and PT, but was undetectable in native VSMC and PAEC, and very low in freshly isolated PT cells.

MATERIALS AND METHODS

Isolation and primary culture of PT cells, VSMC and PAEC

Rabbit kidney proximal tubule (PT) cells were isolated and cultured as described previously [Rose *et al*, 1993] VSMC were isolated from 6 8 weeks old male Wistar rats Aortic sections, from its ventricular origin to the branching of the renal arteries, were rapidly excised and placed in Dulbecco's modified Eagle's medium (Imperial , Hampshire, UK) supplemented with 10 μg/ml gentamycin (DME$^+$) medium The aortas were cleaned of fat and connective tissue, cut longitudinally and segments of the medial smooth muscle layer were peeled away from the adventitial layer and stored in DME$^+$ medium These segments were cut into fine pieces (1 x 1 mm) which were frozen in liquid nitrogen and stored at -80 ° C until RNA isolation For cell culture, tissue pieces were placed in a 5 ml tube and allowed to pelletate by means of gravitation before the medium was replaced by DME$^+$ (1 ml/aorta), supplemented with 1 mg/ml (405 U/mg) collagenase (Sigma, St Louis, MO, USA), 0 5 mg/ml elastase (Boehringer Mannheim, Mannheim, Germany) and 0 5 mg/ml soy-bean trypsin inhibitor (Worthington Biochemical Corporation, Freehold, NJ, USA) The tube was placed in a roll over rotor (~30 rpm) at 37 °C for ~ 1 hour VSMC were isolated by enzymatic dissociation during this period and digestion was stopped by adding 10 % (vol/vol) fetal calf serum (FCS) (Serva, Heidelberg, Germany) Cells were collected by centrifugation (5 min, 200 x g) After aspiration of the digestion medium the cells were resuspended in 2 5 ml/aorta DME$^+$, supplemented with 10 % (vol/vol) FCS, 1% 100 x non essential amino acids (Gibco, Paisley, UK) and 28 mM L glutamine and subsequently seeded on 6-wells plates (4 aortas/plate) coated with fibronectin extracted from human plasma (0 1 μg/cm^2, kindly provided by the Central Laboratory for Blood Transfusion, Amsterdam, The Netherlands) Cells were cultured in a humidified incubator equilibrated with 5% CO, 95% air at 37 °C Medium was changed after 3 days subsequently every 48 hours and the day before experiments were performed Cells were used after 7 days in culture

Endothelial cells were isolated from the aortas of adult pigs and obtained from the slaughterhouse Aortas were cleaned of connective tissue, cut open longitudinally and the inner wall of the aorta was intensively squirted with PBS from a siphon to remove adhering blood cells For cell culture the endothelial layer was removed by incubation of the aortic wall with 1 mg/ml collagenase (Boehringer Mannheim, Mannheim, Germany) for 5 min Subsequently, cells were collected in RPMI medium (Gibco, Paisley, UK) to which 10% (vol/vol) FCS was added to inhibit enzyme digestion Cells were spun down and resuspended in EC medium containing an 1 1 mixture of RPMI medium and 199 medium (Flow Laboratories, Irvine, Scotland) supplemented with 5 % (vol/vol) FCS, gentamycin (10 μg/ml), endothelial cell growth factor (30 μg/ml) (kindly provided by Solvay Duphar, Weesp, The Netherlands), L glutamine (2 mM) and pyruvate (1 mM) Cells were seeded on fibronectin coated (0 1 μg/cm^2) culture flasks in EC medium Passages 2 to 5 were used for experiments For RNA isolation from freshly isolated cells, the endothelial layer was removed with a plastic scraper (Costar, Cambridge MA, USA) and frozen in liquid nitrogen and stored at 80 ° C until use

Bumetanide-sensitive $^{86}Rb^+$ uptake

$Na^+/K^+/2Cl$ cotransport activity in confluent monolayers of cultured cells was measured as the bumetanide sensitive $^{86}Rb^+$ uptake in the presence of 1 mM ouabain to inhibit the Na^+/K^+-ATPase as described previously [Raat *et al*., 1994] To measure cotransport activity in cell suspensions, cells were directly resuspended (~ $5\ 10^6$ cells/ml) after cell isolation or trypsinization in isotonic (300 mosM) Krebs Henseleit buffer (KHB) which contained (in mM) 110 NaCl, 5 KCl, 2 NaH_2PO_4, 1 2 $MgSO_4$ 10 Na-acetate, 4 L lactate, 10 D-glucose, 1 L-alanine and 1 $CaCl_2$, 20 HEPES, calibrated with TRIS to pH 7 4 Cell viability was determined by trypan blue exclusion To 300 μl of isotonic KHB medium containing 1 mM ouabain, in the presence or absence of 10 μM bumetanide, a volume of 100 μl cell suspension was added After pre incubation of 15 min at 37 °C, 400 μl KHB medium with a osmolarity of 300 or 700 mosM (isotonic medium plus 400 mM mannitol) and containing 10 μCi ^{86}RbCl per ml was added resulting in a final osmolarity of 300 and 500 mosM respectively $^{86}Rb^+$ uptake was stopped after a 3 min incubation by adding 3 ml of ice-cold stop buffer consisting of (in mM) 140 KCl, 5 $BaCl_2$, 15 tetraethylammonium chloride, 1 ouabain and 10 μM bumetanide Subsequently the cells were washed and centrifugated twice for 5 min at 200 x g Cells were lysed by adding 0 5 ml of 0 05% (vol/vol) sodium dodecyl sulphate to each dried cell pellet and radioactivity was determined by liquid scintillation counting For PT cells a mean protein content of 350 μg/ml cell suspension was determined from 2 samples $Na^+/K^+/2Cl$ cotransport activity was expressed as nmol Rb^+ mg protein^{-1} min^{-1}

Northern blot analysis

Total RNA was extracted from freshly isolated PT cells and from 5 day old confluent PT monolayers as described by Chomczynski and Sacchi [1987] Poly $(A)^+$ mRNA was extracted from VSMC and PAEC by using a Quickprep® mRNA purification kit (Pharmacia Biotech, Milwaukee, WI USA) Samples were processed as described in detail by Delpire *et al* [1994] For hybridization a 1200 bp (nucleotides 989 2188) PCR fragment from the 4 7 kb cDNA clone mBSC2 was used [Delpire *et al* 1994]

Statistics

All $^{86}Rb^+$ uptake experiments measurement were performed in duplicate using cells derived from at least three isolations Statistical significance was determined by one way analysis of variance (ANOVA) Data is presented as the mean ± SE

RESULTS

$Na^+/K^+/2Cl^-$ cotransport activity in PT cells

Uptake of $^{86}Rb^+$ was determined in a cell suspension of freshly isolated PT cells at isotonicity (300 mosM) and in the presence of 1 mM ouabain. $^{86}Rb^+$ uptake was not inhibited by 10 μM bumetanide in freshly isolated PT cells. In trypsinized cultured cells, $^{86}Rb^+$ uptake amounted to 5.3 ± 0.4 nmol $^{86}Rb^+$. mg protein^{-1}. min^{-1} in the absence of bumetanide and was inhibited by 53% in the presence of bumetanide (Fig. 1). Since $Na^+/K^+/2Cl^-$ cotransport could be silent in freshly-isolated cells under isotonic conditions, these cells were incubated in hypertonic (500 mosM) medium. This manoeuvre did not uncover bumetanide-sensitive ^{86}Rb uptake (Table 1). In addition, the presence of NaF (10 mM) or calyculin A (1 μM) conditions which activate the transporter in cultured PT cells as previously shown [Raat *et al.*, 1994], had no additional effect on $^{86}Rb^+$ uptake in freshly isolated PT cells (Table 1).

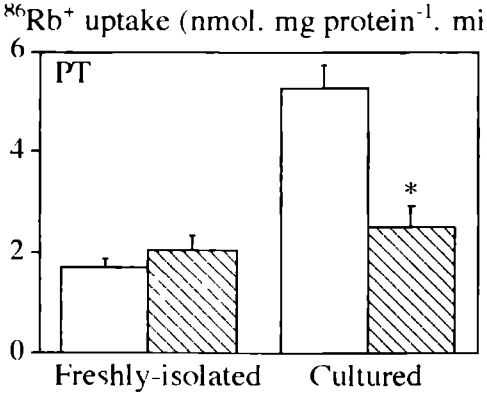

Figure 1
$^{86}Rb^+$ uptake in freshly isolated and trypsinized cultured rabbit proximal tubule cells in 300 mosM medium containing 1 mM ouabain and in the presence (hatched bars) or absence of 10 μM bumetanide (open bars). Values are means ± SE of at least 3 preparations (* $P < 0.05$).

Table 1. $Na^+/K^+/2Cl^-$ cotransport activity in freshly isolated proximal tubule cells

	Ouabain-insensitive $^{86}Rb^+$ uptake (nmol. mg protein^{-1}. min^{-1})		
Incubation condition	- bumetanide	+ bumetanide	n
isotonic	1.7 ± 0.2	2.0 ± 0.3	12
hypertonic	2.3 ± 0.3	2.5 ± 0.4	9
hypertonic + NaF (10 mM)	2.4 ± 0.5	2.8 ± 0.6	8
hypertonic + calyculin (1 μM)	2.3 ± 0.3	2.2 ± 0.4	8

Ouabain-insensitive ^{86}Rb uptake (nmol mg protein^{-1} min^{-1}) in freshly-isolated proximal tubule cells in the absence and presence of 10 μM bumetanide under isotonic (300 mosM) and hypertonic (500 mosM) conditions. Values are means ± SEM of at least 3 separate isolations

Na^+ /K/2Cl cotransport activity in VMSC and PAEC.

$Na^+/K^+/2Cl^-$ cotransport activity was observed in confluent monolayers of cultured vascular smoothmuscle cells (VSMC) from rat aorta in the presence of ouabain (1 mM). At isotonicity ouabain-insensitive and bumetanide-sensitive $^{86}Rb^+$ uptake was 3.5 ± 0.4 nmol. mg protein $^{-1}$. min (n=7) and significantly increased at hypertonic (500 mosM) conditons to 4.0 ± 0.4 nmol. mg protein $^{-1}$. min. In freshly isolated VSMC no bumetanide sensitive $^{86}Rb^+$ uptake was

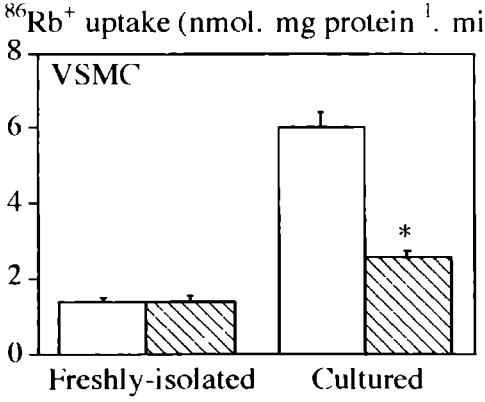

Figure 2
$^{86}Rb^+$ uptake in freshly isolated and cultured vascular smooth muscle cells in 300 mosM medium containing 1 mM ouabain with (hatched bars) or without (open bars) 10 μM bumetanide. Values are means ± SE of at least 3 preparations (* $P < 0.05$).

measurable (Fig.2). $Na^+/K^+/2Cl^-$ cotransport activity was also studied in confluent monolayers of cultured porcine aortic endothelial cells (PAEC). At isotonicity the ouabain-insensitive and bumetanide-sensitive $^{86}Rb^+$ uptake in PAEC amounted to 1.3 ± 0.2 nmol. mg protein $^{-1}$. min (n=14) and hypertonicity (500 mosM) stimulated cotransport activity to 3.2 ± 0.3 nmol. mg.protein $^{-1}$. min (n=14). As with VSMC, no bumetanide-sensitive $^{86}Rb^+$ uptake was detectable in freshly isolated aortic endothelial cells (Fig.3).

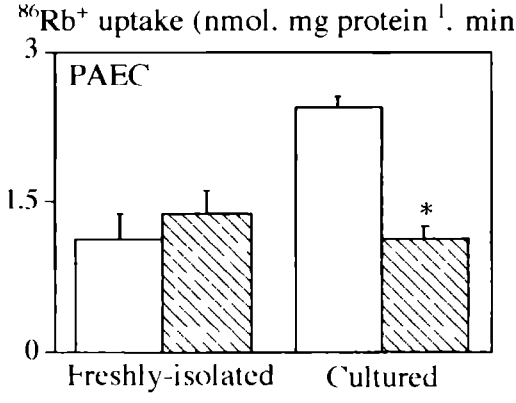

Figure 3
$^{86}Rb^+$ uptake in freshly isolated and cultured porcine aortic endothelial cells in 300 mosM medium containing 1 mM ouabain with (hatched bar) or without (open bar) 10 μM bumetanide Values are means ± SE of at least 3 preparations (* $P < 0.05$)

Northern blot analysis

For hybridization, a ^{32}P labeled 1200 bp PCR fragment from the 4.7 kb cDNA clone mBSC2 described by Delpire *et al.* [1994] was used. After 6 hr exposure, a major transcript at 6.5 kb was detected with this probe on a Northern blot containing 10 μg of total RNA extracted from cultured rabbit proximal tubule (PT) cells (Fig. 4, lane 1). As previously shown by Delpire *et al.* [1994] this 6.5 kb band represents an extension of the 3'-untranslated sequence of mBSC2 (4.7 kb) with an alternative polyadenylation site. In contrast, the autoradiograph showed no band in the corresponding lane on the Northern blot containing equal amounts of total RNA extracted from freshly-isolated rabbit PT cells (Fig. 4, lane 2). Only after a 72 hr exposure a faint signal was obtained from the freshly-isolated rabbit PT cells (data not shown).

In addition, mRNA samples (5 μg) of freshly-isolated and primary cultured VSMC were screened for the presence of the 6.5 kb transcript. Northern blot analysis revealed a 6.5 kb transcript in primary cultures of VSMC, whereas no band was detected in the samples obtained from freshly-isolated VSMC (Fig. 4, lane 3 and 4). A similar difference in BSC2 expression was observed between cultured and freshly-isolated PAEC (Fig. 4, lane 5 and 6).

DISCUSSION

In the present study we showed for three different tissues (PT, VSMC, PAEC) that the expression of a putative mammalian $Na^+/K^+/2Cl^-$ cotransporter BSC2 [Delpire *et al.*, 1994] is very low in the native tissue while expression is dramatically increased in primary cultures. For the three cell types the expression of the cotransporter as determined by Northern analysis correlated with bumetanide-sensitive $^{86}Rb^+$ uptake rates. Recently, Alvarez and Candia [1994] demonstrated the absence of bumetanide-sensitive $^{86}Rb^+$ uptake in intact bovine lenses while after culturing $Na^+/K^+/2Cl^-$ cotransport became detectable. Our observation suggests that expression of BSC2 is linked to cell proliferation.

Until now, $Na^+/K^+/2Cl^-$ cotransport activity in VSMC [O'Donnell and Owen, 1988; Orlov *et al.*, 1992a; Orlov *et al.*, 1992b] and PAEC [O'Donnell, 1991; Klein *et al.*, 1993] has only been studied in cultured cells since measurements in native tissues are technically difficult [Orlov *et al.*, 1992a]. The recent cloning of genes encoding several isoforms of $Na^+/K^+/2Cl^-$ cotransport [Gamba *et al.*, 1994;

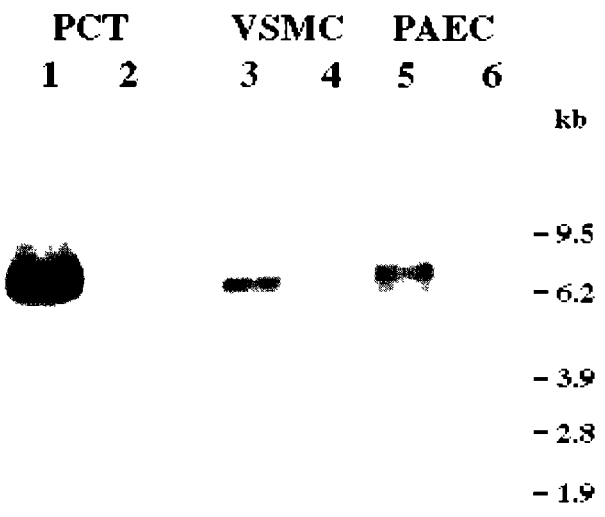

Figure 4

Northern membranes containing 10 μg of total RNA from cultured (lane 1) and freshly isolated (lane 2) rabbit proximal tubule cells (PT) (autoradiograph exposure time was 6 h), 5 μg of poly(A)$^+$ mRNA isolated from cultured (lane 3) and freshly isolated (lane 4) rat vascular smooth muscle cells (VSMC) or 5 μg of poly(A)$^+$ mRNA isolated from cultured (lane 5) and freshly isolated (lane 6) porcine aortic endothelial cells (PAEC) (autoradiograph exposure time was 72 hr) hybridized with the ^{32}P-labelled 1200 bp PCR fragment of mBSC2 [Delpire *et al.*, 1994]. Membranes were washed at high stringency (65 °C, 30 mM NaCl).

Haas, 1994; Xu *et al.*, 1994], however, provides for studying cotransport expression in the native tissue. Differences in $Na^+/K^+/2Cl^-$ cotransport rates determined in cultured VSMC of SHR and WKY have been implicated in the pathogenesis of essential hypertension [Orlov *et al.*, 1992a]. Furthermore, Blaustein [1977] postulated that a disturbance in Na^+ homeostasis could reduce Ca^{2+} extrusion via Na^+/Ca^{2+} exchange, resulting in a rise in vascular tone. In addition, in cultured cells $Na^+/K^+/2Cl^-$ cotransport is regulated by several vasoactive substances which suggests a role in regulation of vascular tone [O'Donnell and Owen, 1986; Owen and Ridge, 1989; Owen *et al.*, 1990]. However, our results raise a serious question whether $Na^+/K^+/2Cl^-$ cotransport in vascular smooth muscle and endothelial cells in intact blood vessels contributes significantly to Na^+, K^+ and Cl^- influx in these cells. If $Na^+/K^+/2Cl^-$ cotransport activity is linked to cell proliferation, as suggested by our observation, it might well be that differences in cotransport activity in VSMC of SHR and WKY are

due to the difference in growth rate that have been reported for the two cell types [Scott-Burden *et al.*, 1989]

There are several studies that support a role for $Na^+/K^+/2Cl^-$ cotransport in cell proliferation. Recently, Delpire and Gullans [1994] showed that cotransport activity decreased during erythroid differentiation. Previous studies also reported that $Na^+/K^+/2Cl^-$ cotransport can be activated by α-thrombin, EGF, TPA and 8-bromo-cGMP [Owen, 1984; Paris and Pouysségur, 1986]. A role for Na /K /2Cl cotransport in cell proliferation is further supported by the finding that $Na^+/K^+/2Cl^-$ cotransport was increased in hypertrophied VSMC [Tseng and Berk, 1992] and that bumetanide inhibited proliferation of endothelial cells [Panet *et al.*, 1994]. When Na /K /2Cl cotransport is needed in cell volume regulation during cell growth, its increase in activity could be a consequence of rather than a cause of hypertrophy observed in SHR derived VMSC. Also in PT cells expression of $Na^+/K^+/2Cl^-$ cotransport could be a consequence of the highly proliferative state of PT cells after seeding. Inhibition of $Na^+/K^+/2Cl^-$ cotransport, however, does not prevent DNA synthesis [Paris and Pouysségur, 1986, Vandewalle *et al.*, 1993]. Since all of these studies have been carried out with cultured cells, it is possible that cotransport is only essential for cell proliferation and that expression of cotransport is suppressed in differentiated cells [Panet *et al.*, 1994]. Our observation on BSC2 expression in cultured cells could also hold for other transport systems like for example Na /H^+ exchange. In the differentiation process of mouse erythroleukemia cells, mRNA levels of the Na^+/H^+ exchanger (NHE-1) increased several fold together with an increase in mRNA encoding band 3 protein [Delpire and Gullans, 1994]. Like Na /K /2Cl cotransport, Na^+/H^+ exchange has been shown to be activated by growth factors [Grinstein *et al.*, 1989] and to be involved in volume recovery after cell shrinkage [Hoffmann and Simonsen, 1989]. In conclusion the present study indicates that caution is needed when conclusions on regulation of $Na^+/K^+/2Cl^-$ cotransport derived from cultured cells are extrapolated back to the tissue of origin, especially when the occurrence of cotransport in the fully differentiated tissue is not well established.

CHAPTER 7

General Discussion and Summary

In this thesis several aspects of cell volume regulation in rabbit proximal tubule (PT) cells and rat vascular smooth muscle cells (VSMC) in primary culture were studied. Although cell volume regulation is important in all cell types, these specific cell types were chosen for particular reasons. Across the PT epithelium the rate of salt and water transport is very high [Lohr and Grantham, 1986] and, therefore, in- and efflux of ions have to be in balance in order to guarantee a constant cell volume. During conditions that in- or efflux suddenly change, cell volume regulatory mechanisms will be needed to protect the cell from any damage. To better understand the process of volume regulation in PT cells, the course of cell volume after cell volume perturbation was followed by three different techniques (chapter 5). Besides, changes in intracellular calcium concentration ($[Ca^{2+}]_i$) and intracellular pH (pH_i) that might occur concomitantly and could mediate regulation of transport were investigated (chapter 2). The possible contribution of $Na^+/K^+/2Cl^-$ cotransport to cell volume recovery after cell shrinking was determined by measuring cell volume (chapter 5) as well as measuring K^+ influx by $^{86}Rb^+$ uptake studies (chapter 4). In vascular smooth muscle cells of spontaneously hypertensive rats (SHR) different rates of $Na^+/K^+/2Cl^-$ cotransport have been reported when compared to normotensive controls [Tokushige *et al.*, 1986; O'Donnell and Owen, 1988; Orlov *et al.*, 1992]. According to a hypothesis by Blaustein *et al.* [1977] increases in intracellular Na^+ will interfere with cellular Ca^{2+} homeostasis via reversed operation of the $3Na^+/Ca^{2+}$ exchanger. Elevation of $[Ca^{2+}]_i$ is known to increase vascular tone which is one of the characteristics of essential hypertension [Bohr and Webb, 1988; Orlov *et al.*, 1992a]. In chapter 3 $[Ca^{2+}]_i$ was measured in single VSMC, isolated from SHR and from normotensive Wistar Kyoto (WKY) rats by fluorescent ratio imaging.

A transient elevation of $[Ca^{2+}]_i$ was measured after hypotonic cell swelling in both PT cells (chapter 2) and VSMC (chapter 3) which is in agreement with results reported in other cell types [McCarty and O'Neil, 1992; Bibby and McCulloch, 1994]. The increase in $[Ca^{2+}]_i$ is involved in activation of K^+ and Cl^- channels leading to efflux of KCl and water [McCarty and O'Neil, 1992] resulting in a decrease of cell volume. The increase in $[Ca^{2+}]_i$ in PT cells was found to be both from extracellular and intracellular origin. During hypotonic cell swelling PT cells slowly acidified which could not be attributed to a decrease in the rate of Na^+/H^+ exchange or an increase in the rate of Cl^-/HCO_3^- exchange.

Responses in pH_i are less univocal as changes in $[Ca^{2+}]_i$ since both cell acidification [Livne *et al.*, 1987; Star *et al.*, 1992] and alkalinization [Beck *et al.*, 1992] have been reported upon cell swelling in various types of cells. The decrease in pH_i during hypotonic swelling of PT cells was not directly coupled to the simultaneous elevation in $[Ca^{2+}]_i$ since prevention of this Ca^{2+} spike by the Ca^{2+}-chelator BAPTA did not affect cell acidification. Therefore, pH_i could besides $[Ca^{2+}]_i$ play a role in the signal transduction behind cell volume regulation. The conductance of K^+ channels has been described to be lowered by a decrease in pH_i [Beck *et al.*, 1992]. Since the swelling induced cell acidification is slow compared to the response in $[Ca^{2+}]_i$ the role of pH_i could be a slow inactivation of K^+ efflux after completion of the recovery of cell volume near resting values. This hypothesis is, however, difficult to test since in an experimental approach to clamp pH_i the ionophore nigericin and a high extracellular potassium concentration is used but this procedure is not applicable in cell swelling experiments as it prevents K^+ efflux.

Like PT cells, VSMC showed a Ca^{2+} transient after incubation in hypotonic medium, which slowly decreased and reached a sustained level above the initial resting $[Ca^{2+}]_i$. VSMC from SHR had a higher basal $[Ca^{2+}]_i$ and, in addition, the swelling induced Ca^{2+} peak as well as the sustained $[Ca^{2+}]_i$ level during cell shrinkage were both higher in VSMC isolated from SHR (chapter 3). The increased basal $[Ca^{2+}]_i$ in VSMC of SHR is in agreement with other studies [Sugiyama *et al.*, 1990; Bendhack *et al.*, 1992; Asano *et al.*, 1993] and may be explained by a difference in cellular Ca^{2+} homeostasis in VSMC from SHR. The higher sustained $[Ca^{2+}]_i$ level in VSMC of SHR during cell shrinkage might be a consequence of larger Na^+ influx by cell volume recovery mechanisms as the Na^+/H^+ exchanger or the $Na^+/K^+/2Cl^-$ cotransporter. The latter mechanism was excluded since no difference in cotransport activity could be detected in VSMC from SHR and WKY.

Relatively few studies reported changes in $[Ca^{2+}]_i$ and pH_i after hypertonic cell shrinkage. In PT cells as well as in VSMC an abrupt decline in $[Ca^{2+}]_i$ was measured after cells were incubated in hypertonic media. In PT cells the decrease in $[Ca^{2+}]_i$ is probably caused by increased Ca^{2+} efflux, but is hard to prove due to the lack of specific inhibitors of the plasma membrane Ca^{2+}-ATPase. Recently a similar decrease in $[Ca^{2+}]_i$ was reported to occur in human gingival fibroblasts [Bibby and McCulloch, 1994]. It is possible that the decrease in $[Ca^{2+}]_i$ has a direct effect on transport mechanisms involved in cell volume regulation. In this

thesis in which $^{86}Rb^+$ uptake was used to assess $Na^+/K^+/2Cl^-$ cotransport activity it was shown that cotransport activity increased in PT cells in a Ca^{2+}-free medium (chapter 4) while high $[Ca^{2+}]_i$ was inhibitory. Similar results have been reported for other cell types [Haas, 1989] which supports this notion. After hypertonic cell shrinkage, PT cells showed a fast alkalinization and this increase might be explained by an increased rate in Na^+/H^+ exchange that is activated by cell shrinkage as was demonstrated for other cell types [Hoffmann and Simonsen, 1989]. This would indicate that cell alkalinization can be considered as a result of rather than an initiating factor in cell volume regulation. In PT cells alkalinization was only partially reduced after inhibition of Na^+/H^+ exchange and only when cell shrinkage was preceded by hypotonic preincubation. This result indicates that Na^+/H^+ exchange has to be activated first and that an isoform of the Na^+/H^+ exchanger, NHE-2, with reduced amiloride sensitivity which is present in the apical membrane of polarized cells [Rosskopf *et al.*, 1993] might be responsible for the shrinkage-induced alkalinization.

In PT cells the changes in $[Ca^{2+}]_i$ and pH_i observed after cell volume perturbation with anisosmotic solutions were opposite to each other. When during isotonic conditions either $[Ca^{2+}]_i$ or pH_i was changed, the other parameter was mutually affected but in an opposite way than in anisosmotic conditions. These observations are hard to explain, but they indicate that changes in $[Ca^{2+}]_i$ and pH_i induced by cell volume perturbation are clearly dissociated and are independent phenomena.

The presence of $Na^+/K^+/2Cl^-$ cotransport was demonstrated in rabbit PT cells in primary culture (chapter 4). Cotransport activity was found to be dependent on medium osmolarity, $[Ca^{2+}]_i$ and pH_i. In addition, PKA had an inhibitory effect on cotransport, whereas the phosphatase inhibitor calyculin and the G protein activator NaF were found to stimulate cotransport activity. A role for this cotransporter in RVI is likely since hypotonicity almost totally inhibited cotransport activity while hypertonicity increased its activity. RVI in PT cells was studied by pre-incubation in hypotonic medium since in several cell types RVI does only occur when the cells underwent a RVD [Hoffmann and Simonsen, 1989; Lewis and Donaldson, 1990]. In initial cell volume measurements, using PT cells loaded with fluorescent dyes, the question whether RVI does occur in PT cells could not unambiguously be answered (chapter 5). Later studies, in which cell volume was measured using the more reliable technique of automatic cell thickness monitoring, corroborated that RVI was absent in rabbit PT in primary culture (chapter 5).

In earlier reports the presence of RVI in isolated proximal tubules has been reported, but contradictory results were obtained. Lohr and Grantham [1986] showed that tubules maintain their volume when the medium osmolarity was gradually increased to 360 mOsm, whereas a hypertonic shock of similar magnitude did not provoke a RVI response. Kirk *et al.* [1987] were also not able to demonstrate RVI in proximal tubules after a hypertonic shock. Rome *et al.* [1989] reported that tubules incubated in medium that in addition to glucose contained acetate, alanine, lactate and citrate demonstrated a partial RVI when medium osmolarity was rapidly increased. An explanation for this observation was attributed to the fact that Webb *et al.* [1986] had shown that these substances are important for maximal transcellular transport of solute and water. All these components, except citrate, were present in the media used in this thesis, but no RVI was observed. In contrast, Linshaw *et al.* [1992] showed a RVI response when tubules were reperfused with isotonic medium after hypotonicity without addition of extra substrates to the medium. Although $Na^+/K^+/2Cl^-$ cotransport activity was present in PT cells in culture its activity may not be sufficient to alter cell volume significantly Alternatively, the osmotic gradient to which the cells were exposed could have been too far from the physiological situation. However, in Coulter counter experiments RVI was neither observed in cultured PT cells that were exposed to 400 mosM medium instead of 500 mosM. Finally, it is possible that the functional expression of transporters involved in RVI is decreased during prolonged culturing.

In the literature there is no evidence for the presence of $Na^+/K^+/2Cl^-$ cotransport in fully differentiated proximal tubular cells. Only in one preliminary study by Witthembury *et al.* [1992] this cotransport was uncovered in proximal straight tubules after a hyperosmotic (30 mosM urea) shock. Chapter 6 describes that cotransport activity could not be measured in freshly isolated PT cells when determined by $^{86}Rb^+$ uptake studies. These results were confirmed when RNA isolated from freshly isolated and cultured PT was subjected to Northern blotting and screened with a cDNA probe directed against BSC2 [Delpire *et al.*, 1994], an isoform of $Na^+/K^+/2Cl^-$ cotransport. The quantity of RNA encoding BSC2 was barely detectable in freshly isolated PT whereas the transcript could be clearly detected in PT cells in primary culture A similar pattern was found in freshly isolated and cultured VSMC and porcine aortic endothelial cells (PAEC). In these cells, several vasoactive substances have been reported to modulate $Na^+/K^+/2Cl^-$ cotransport activity [O'Donnell and Owen, 1986; O'Donnell, 1989a, Owen and

Ridge, 1989; Owen *et al.*, 1990], suggesting a role in the regulation of vascular tone. Cotransport has been reported to be disturbed in VMSC from SHR possibly resulting in alteration of Na^+ influx which via $3Na^+/Ca^{2+}$ exchange could affect cellular Ca^{2+} homeostasis. As cellular Ca^{2+} is an important factor in the control of vascular tone, these studies suggested a role for cotransport in the pathogenesis of hypertension [Tokushige *et al.*, 1986; O'Donnell and Owen, 1988; Orlov *et al.*, 1992a]. The results described in chapter 6 raise the question to what extent $Na^+/K^+/2Cl^-$ cotransport contributes to Na^+ influx in the fully differentiated tissue.

The difference in expression of $Na^+/K^+/2Cl^-$ cotransport between freshly isolated and cultured VSMC, PAEC and PT cells suggest that cell proliferation is a prime regulator of $Na^+/K^+/2Cl^-$ cotransport expression. Recently, Alvarez and Candia [1994] reported similar results in bovine lens epithelial cells, based on $^{86}Rb^+$ uptake studies. They provided three possible explanations for the absence of cotransport in the intact bovine lens. First, cotransport expression is associated with the proliferation state of the cells. Second, the activity of cotransport is better detectable in cultured cells lacking apical-basolateral membrane differentiation due to culturing on impermeable substrate. Third, the cotransporter is present in the differentiated tissue in a latent form. However, as described in chapter 4 cotransport in PT cells could not be activated by cell volume perturbation in a hypertonic medium or by adding NaF or calyculin, agents that activate cotransport in cultured PT cells

There are several indications that $Na^+/K^+/2Cl^-$ cotransport is involved in cell volume regulation during cell growth. When erythrocytes mature, a reduction in their cell volume is observed and Delpire and Gullans [1994] showed that this shrinkage coincides with a 90% decline in $Na^+/K^+/2Cl^-$ cotransport activity when mouse erythroleukemia cells differentiated. Several studies have reported a decrease in cotransport activity in quiescent cells and in PT cells also a small decrease in cotransport activity was observed after hormonal deprivation (chapter 4). Furthermore, $Na^+/K^+/2Cl^-$ cotransport can be activated by hormones and growth factors like epidermal growth factor and a-thrombin, cGMP and the phorbol ester TPA [Owen, 1984; Paris and Pouysségur, 1986]. Panet *et al.* [1994] showed that the low activity of $Na^+/K^+/2Cl^-$ cotransport in quiescent subconfluent vascular endothelial cells was dramatically stimulated by fibroblast growth factor and that bumetanide reversibly inhibited cell proliferation. Expression of the Ha-ras oncogene in NIH fibroblasts was shown to stimulate Na^+/H^+ exchange and $Na^+/K^+/2Cl^-$ cotransport and cell volume had increased compared to fibroblasts

that lack the oncogene [Lang *et al*., 1992]. Tseng and Berk [1992] found that cotransport is also increased in hypertrophied VSMC. Differences in growth rate have been reported between VSMC from SHR and WKY rats [Scott-Burden *et al*., 1989] and it could be that a difference in growth rate is responsible for this difference in cotransport activity. Variation in growth conditions might explain why in some studies an increase [Tokushige *et al*., 1986; Orlov *et al*., 1992b], in another a decrease [O'Donnell and Owen, 1988] and no difference in $Na^+/K^+/2Cl^-$ cotransport activity in VSMC of SHR was measured in the study described in chapter 4.

In the following paragraph several remarks concerning cell volume regulation in general are discussed. The process of cell volume regulation can be studied in several ways depending on how the cell volume is perturbed, the time period needed for recovery and the kind of osmolyte which is involved. In this thesis studies were limited to inorganic ion transport systems which are activated instantaneously upon cell volume perturbation by anisosmotic solutions. Another way to change cell volume is by incubation in isosmotic media in which Na^+ or Cl^- are replaced by an impermeable solute or a solute that accumulates intracellularly, resulting in cell shrinkage or cell swelling, respectively. It is difficult to decide which method is superior since both methods are far from the physiological situation. Macknight *et al*. [1994] argue that to better understand the cell volume recovery mechanisms *in vivo* the experimental conditions should resemble the physiological situation. Accordingly, to their opinion acute changes in osmolarity should be avoided and osmolarity should gradually change with time. They also argue that the time course of volume changes in tissue develops over minutes to hours and completely differs from experiments in which cell volume was acutely perturbed with anisosmotic media. Strange [1994] supports this opinion and suggests that cells may have multiple volume sensors that could activate volume recovery systems depending on the osmotic gradient. In addition, he suggests that cells can differentiate between the way their cell volume is altered. The use of large osmotic shocks might also explain the observation that cells do not always recover their volume to initial values after a sudden anisosmotic shock [Hoffmann and Simonsen, 1989]. The transport rate in this case may be inadequate when greater osmotic gradients are applied. Therefore $Na^+/K^+/2Cl^-$ cotransport in PI cells could play a role in maintenance of cell volume during for example cell growth whereas it is insufficient to compensate for cell shrinkage after hypertonic shrinkage. One should realise, however, that it

will be difficult to detect minute differences in cell volume and accurately measure associated intracellular signals Therefore, improvement in the resolution of cell volume measurement techniques will certainly be needed to overcome the drawbacks as mentioned above Much of the knowledge on the contribution of organic osmolytes to volume recovery stems from studies on invertebrates [Chamberlin and Strange, 1989] In vertebrate cells inorganic solutes have shown to be the most important [Hoffmann and Simonsen, 1989] A recent study, however, by Jackson and Strange [1993] reports the discovery of a volume-sensitive anion channel that is also permeable for organic osmolytes This finding suggests that the contribution of organic osmolytes to cell volume recovery in vertebrate cells may be greater than thought until now and certainly will have effect on the way cell volume is studied in the future In general, regulation of volume by organic osmolytes is relatively slow compared to regulation by inorganic osmolytes and may require many hours to reach completion In this process even gene translation and protein synthesis may be affected [Strange, 1994].

In conclusion, hypotonic swelling in cultured rabbit PT cells induced a transient increase in $[Ca^{2+}]_i$ together with a slow cellular acidification Conversely, hypertonic shrinkage resulted in a rapid and sustained decline in $[Ca^{2+}]_i$ and a fast alkalinization The changes in $[Ca^{2+}]_i$ and pH_i under anisosmotic conditions appeared to be independent phenomena since an opposite relation was measured at isotonic conditions VSMC of SHR had a higher basal $[Ca^{2+}]_i$ level and, in addition, the swelling induced Ca^{2+} spike and the sustained $[Ca^{2+}]_i$ levels during cell shrinkage were higher than in VSMC of control WKY rats In PT cells, changes in the intensity of trapped fluorescent dyes could not be used as an estimate for alterations in cell volume With the more reliable method of automatic cell height measurement a partial RVD was observed in PT cells after hypotonic swelling, while no RVI could be demonstrated after subsequent hypertonic incubation $Na^+/K^+/2Cl^-$ cotransport was identified in rabbit PT cells in primary culture and its activity was dependent on medium osmolarity, $[Ca^{2+}]_i$, pH_i, and the state of phosphorylation Cotransport activity was not detectable in freshly isolated PT cells by using $^{86}Rb^+$ uptake studies Northern blotting using a cotransport specific probe confirmed this result as it showed that the transcript was almost absent in RNA samples from freshly isolated PT cells, while it was clearly detectable in PT cells in primary culture These differences in expression of cotransport suggests a correlation with cell growth A similar observation was

made for the expression of cotransport in freshly isolated and cultured VSMC and PAEC. Studies in these cells have pointed to a role for cotransport in the pathogenesis of hypertension, but were all performed in cultured cells. The results from chapter 6 of this thesis however show that caution is needed when the outcome of transport studies in cultured cells are extrapolated to the fully differentiated state occurring in the tissue of the body.

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Samenvatting

Samenvatting

Het vermogen om het celvolume binnen nauwe grenzen constant te houden is een belangrijke fysiologische eigenschap van de meeste cellen. Hierbij spelen verschillende transport- en signaaltransductie processen een rol. Concentratie-veranderingen van metabolieten en signaalstoffen als gevolg van schommelingen in het celvolume kunnen voor de cel levensbedreigend zijn. Door de hoge waterpermeabiliteit van dierlijke cellen wordt het celvolume bepaald door de osmolariteit van het cytoplasma en van de extracellulaire vloeistof. In zoogdieren wordt de osmolariteit van de lichaamsvloeistoffen nauwkeurig op peil gehouden door de nier. De intracellulaire osmolariteit kan echter door verschillende factoren beïnvloed worden, maar de meeste cellen zijn in staat de gevolgen voor het celvolume te compenseren. Enerzijds is er een passieve instroom van zouten en water als gevolg van de aanwezigheid van geladen macromoleculen in het cytoplasma. Anderzijds blijken ook plotselinge veranderingen in het metabolisme, hormoonconcentratie, de membraanpotentiaal en pH_i de osmolariteit van het cytoplasma te kunnen verstoren. Daarnaast is het in epithelia die zout en water transporteren belangrijk dat de in- en uitstroom van zouten op elkaar is afgestemd. Bij een plotselinge verandering van een van deze transportprocessen kan verstoring van het celvolume optreden. Eveneens speelt celvolumeregulatie een rol bij celgroei en celdeling.

De transportprocessen die bij celvolumeregulatie betrokken zijn zullen onder normale omstandigheden nauwelijks actief zijn. Door de cellen te laten zwellen in een hypotoon medium of te laten krimpen in een hypertone oplossing worden deze transportprocessen geactiveerd. Het proces van volumeregulatie kan grofweg in drie fasen worden onderverdeeld. Allereerst zal de cel in staat moeten zijn de volumeverandering te detecteren. Dit is een proces waarvan nog niet veel bekend is en dat buiten het kader van dit proefschrift valt. Vervolgens zal de cel dit signaal moeten verwerken waarbij tal van signaaltransductiemechanismen betrokken zijn en waarvan er in dit proefschrift een aantal aan de orde komen. In laatste instantie worden transportprocessen geactiveerd die zorg dragen voor het herstel van het celvolume. Een van deze transporteiwitten, de $Na^+/K^+/2Cl^-$ cotransporteur, is in dit proefschrift nader bestudeerd en speelt in veel cellen een rol bij het herstel van het celvolume na een volumeafname.

In hoofdstuk 2 is beschreven hoe de intracellulaire calciumconcentratie ($[Ca^{2+}]_i$) en intracellulaire pH (pH_i) in proximale tubuluscellen (PT cellen)

veranderen na een opgedrongen zwelling of krimp. De $[Ca^{2+}]_i$ en pH_i werden gemeten in individuele cellen met behulp van de fluorescente indicatoren fura-2 en BCECF. Onder isosmotische omstandigheden leidt verhoging van de pH_i tot een verhoging van de $[Ca^{2+}]_i$ en een verzuring tot een daling in de $[Ca^{2+}]_i$. Een daling in de $[Ca^{2+}]_i$ op zijn beurt heeft eveneens een daling in pH_i tot gevolg. Deze onderlinge relatie tussen de $[Ca^{2+}]_i$ en pH_i blijkt na verstoring van het celvolume ontkoppeld te worden. Tijdens celzwelling stijgt $[Ca^{2+}]_i$ tijdelijk en tegelijkertijd verzuurt de cel langzaam. Een tegenovergestelde reactie is waarneembaar tijdens celkrimp, waarbij de $[Ca^{2+}]_i$ daalt en de pH_i toeneemt. Wanneer de stijging in $[Ca^{2+}]_i$ wordt voorkomen door de intracellulaire Ca^{2+}-buffer BAPTA treedt nog steeds een verzuring van de cel op.

In hoofdstuk 3 zijn de veranderingen beschreven die optreden in $[Ca^{2+}]_i$ na zwelling of krimp van gekweekte vasculaire gladde spiercellen uit de aorta van de rat. Bestudeerd is of er verschillen zijn tussen gladde spiercellen geïsoleerd uit ratten met een verhoogde bloeddruk (de zogenaamde spontaan hypertensieve rat of SHR) en die uit de controle stam de Wistar Kyoto (WKY) rat. De rustwaarde voor $[Ca^{2+}]_i$ in gladde spiercellen van de SHR was hoger dan die in cellen van de WKY. Vergelijkbaar met de reactie in proximale tubulus cellen treedt in de gladde spiercellen van SHR en WKY ratten na celzwelling een tijdelijke stijging op in $[Ca^{2+}]_i$ die significant hoger is in spiercellen van de SHR. In tegenstelling tot PT cellen stabiliseert de $[Ca^{2+}]_i$ zich in beide gevallen na enkele minuten op een niveau dat boven de uitgangswaarde ligt. Na een hypertone celkrimp daalt de $[Ca^{2+}]_i$, herstelt daarna gedeeltelijk en komt bij gladde spiercellen uit de SHR op een hoger niveau uit dan in cellen afkomstig uit de WKY rat. Met behulp van $^{86}Rb^+$ opname studies is de activiteit van $Na^+/K^+/2Cl^-$ cotransport in gladde spiercellen van SHR en WKY rat bepaald. In tegenstelling tot eerder gepubliceerde gegevens in de literatuur werd geen significant verschil in activiteit van het cotransport tussen beide rattestammen waargenomen.

In hoofdstuk 4 is met behulp van drie verschillende methoden het verloop in celvolume gemeten van gekweekte proximale tubulus cellen na perfusie met hypo- en hypertone buffers. In eerste instantie is geprobeerd om de veranderingen in celvolume af te leiden uit de concentratieveranderingen van de fluorescente indicatoren fura-2 en BCECF tegelijk met de meting van $[Ca^{2+}]_i$ en pH_i. Deze methode gaf geen betrouwbare resultaten en leidde soms tot zeer tegenstrijdige gegevens binnen één experiment. Bepaling van het celvolume met behulp van geautomatiseerde meting van de celdikte leverde wel betrouwbare resultaten op

Direct na incubatie in een hypotoon medium neemt het celvolume snel toe om vervolgens binnen 5 minuten weer terug te keren naar een gestabiliseerd celvolume dat echter nog wel groter is dan het uitgangsvolume Een hierop volgende incubatie in hypertoon medium veroorzaakte een snelle afname van het celvolume die niet gevolgd wordt door een herstel van het celvolume. Vergelijkbare resultaten werden gemeten met behulp van de Coulter counter met een suspensie van PT cellen Echter, de gemeten celzwelling met de Coulter counter is veel groter in vergelijking met de celdikte meting, waarschijnlijk doordat in losse cellen in suspensie het weefselverband is verdwenen en de cel een groter volume kan innemen.

In hoofdstuk 5 is met behulp van $^{86}Rb^+$ opname studies de aanwezigheid van de $Na^+/K^+/2Cl^-$ cotransporteur in gekweekte proximale tubulus cellen aangetoond Tevens is deze cotransporteur verder gekarakteriseerd en is onder andere de betrokkenheid van een aantal signaaltransductie componenten op cotransporteuractiviteit bestudeerd. De transportactiviteit werd gestimuleerd door verhoging van de mediumosmolariteit, een verlaging van de $[Ca^{2+}]_i$, een toename van de pH_i alsmede door remming van phosphataseactiviteit en stimulering van G-eiwitten Daarentegen veroorzaakte een verhoging van de $[Ca^{2+}]_i$ of een verzuring van de cel een remming van de cotransport activiteit Calmidazolium, IPA, staurosporine, 8-BrcGMP hadden allen geen invloed op de cotransport activiteit

In hoofdstuk 6 wordt beschreven dat met behulp van de $^{86}Rb^+$ opnamemethode geen $Na^+/K^+/2Cl^-$ cotransport activiteit kan worden aangetoond in volledig gedifferentieerde en vers geïsoleerde PT cellen Daarom is RNA geïsoleerd uit vers geïsoleerde en uit gekweekte PT cellen. Met behulp van Northern blotting met een voor het cotransport specifieke cDNA probe, is aangetoond dat een transcript aanwezig is in gekweekte PT cellen, maar nauwelijks in gedifferentieerde PT cellen Voor zowel vasculaire gladde spiercellen uit ratteaorta als voor endotheelcellen uit varkensaorta werd een vergelijkbaar resultaat gevonden. Deze resultaten geven aan dat tijdens het kweken van cellen er een grote toename in de expressie van het $Na^+/K^+/2Cl^-$ cotransport optreedt Mogelijk is $Na^+/K^+/2Cl^-$ cotransport betrokken bij de regulatie van het celvolume tijdens celgroei. Indien de activiteit van het $Na^+/K^+/2Cl^-$ cotransport wordt bepaald in gekweekte cellen is dat dus geen goede maat voor de cotransport activiteit in gedifferentieerde cellen in het oorspronkelijke weefsel.

In hoofdstuk 7 zijn de belangrijkste resultaten nog eens op rij gezet en van commentaar voorzien

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Eric Delpire I want to thank you for your willingness to collaborate with us in screening RNA samples of several cells types using your cotransport probe This technique enabled us to strengthen our results obtained from conventional ^{86}Rb uptake studies and pointed to a marked difference in expression of the cotransporter in fresh and cultured cells

Peter Ramakers en Hanneke van Boxtel jullie waren beiden belangrijk als eerste schakel in de experimenten. Als lableverancier van niercellen kon ik op jullie altijd een beroep doen, ook bij grote aantallen Anita Hartog jouw experimentele vaardigheid maakte dat je op verschillende fronten betrokken bent geweest bij mijn onderzoek Zo hielp je met de opname studies, het isoleren van proximale tubuluscellen en gaf je adviezen bij het opzetten van de gladde spiercellen en hielp je mee wanneer er meerdere aorta's tegelijkertijd geïsoleerd moesten worden Uschi Rose bedankt voor je instructie bij het isoleren van verse endotheel cellen, waarbij Maarten de Jong behulpzaam was, en het beschikbaar stellen van gekweekte endotheelcellen. Rianne Tijsen, bedankt voor je hulp bij de opname studies en je gezelligheid, ook buiten het lab en ik vind het leuk dat je als paranimf tijdens de promotie aanwezig wilt zijn.

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In het laatste stadium van mijn promotietijd is het alsnog gelukt om wat verkennende experimenten te doen met celvolumemeting met de Coulter counter Lex Theuvenet van de afdeling Celbiologie stelde ons hun Coulter counter beschikbaar Richard Huyben van de afdeling Tumorimmunologie lukte het vervolgens om het apparaat weer aan de praat te krijgen Richard, bedankt voor je

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Dat het isoleren van voldoende mRNA uit vasculaire gladde spiercellen geen sinecure is in de laatste periode van het onderzoek wel duidelijk geworden. Peter Deen bedankt voor je adviezen en suggesties hieromtrent en Reagan Pieters voor de hulp bij de isolatie.

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CURRICULUM VITAE

Nicolaas Jacobus Henricus (Harold) Raat werd geboren op 17 augustus 1966 in Uitgeest. In 1985 behaalde hij het Atheneum B diploma aan het Petrus Canisius College in Alkmaar. Aansluitend werd aangevangen met de studie Biologie aan de Universiteit van Amsterdam met als afstudeerrichting Medische Biologie. De westenschappelijke stages werden uitgevoerd binnen de afdelingen Celbiologie en Moleculaire Biologie van het Nederlands Kanker Instituut in Amsterdam. Van september 1990 tot september 1994 was hij als onderzoeker in opleiding werkzaam binnen de afdeling Celfysiologie aan de Faculteit der Medische Wetenschappen van de Katholieke Universiteit Nijmegen. Het onderzoek werd gefinancierd door de Nederlandse Organisatie voor Wetenschappelijk onderzoek (NWO). Tijdens deze periode werd het hiervoor beschreven onderzoek verricht onder de begeleiding van Prof. Dr. C.H. van Os en Dr. R.J.M. Bindels.

Sinds 1 maart 1995 is hij als post-doctoraal onderzoekmedewerker verbonden aan de afdeling Farmacologie van het Cardiovascular Research Institute Maastricht (CARIM). Binnen de groep van Dr. J.G.R. De Mey zal hij zich gaan bezighouden met de gelijktijdige meting van spierspanning en verandering in calciumconcentratie tijdens de samentrekking van kleine bloedvaatjes. De invloed van verschillende signaaltransductie componenten op de koppeling tussen calcium en spierspanning in bloedvaatjes zal hierbij nader worden onderzocht.